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Award Number: DAMD17-97-1-7224

TITLE: Characterization of the Breast Cancer Susceptibility Gene, BRCA2

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REPORT DATE: September 2000

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2000	Final (16 Aug 97 - 15 Aug 00)

4. TITLE AND SUBTITLE
Characterization of the Breast Cancer Susceptibility Gene, BRCA2

5. FUNDING NUMBERS
DAMD17-97-1-7224

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

Estrogen promotes DNA synthesis in breast epithelial cells via its interaction with the estrogen receptor. The primary mode of estrogen action has been considered to be through transcriptional activation of genes containing estrogen response elements. Alternatively, estrogen may stimulate cell cycle progression by activating the MAPK cascade.

Specific small molecule inhibitors of MAPK and PI3-kinase activity were used to determine the influence of these cascades on estrogen-mediated mitogenesis. Inhibitors of both cascades decreased the fraction of cells entering DNA synthesis after treatment with 17β-estradiol. These compounds did not inhibit expression of myc or fos, but did prevent the accumulation of cyclin D1. Interestingly, the downstream targets of these pathways were not activated over basal levels in response to hormone treatment. This suggests that estrogen initiates mitogenesis by regulating transcription, but signaling pathways may regulate subsequent events.

The minimal concentrations of estrogen sufficient to induce mitogenesis or transcription were shown to be the same, implying that these two activities cannot be uncoupled. Cumulatively, these studies indicate that estrogen initiates cell cycle progression by stimulating the transcription of immediate early genes and that the action of basal levels of MAPK and PI3-K are essential at, or prior to, the G1 checkpoint.

14. SUBJECT TERMS
Breast Cancer, Estrogen, Mitogen-Activated Protein Kinase, Phosphatidylinositol 3-Kinase, Cell Cycle

15. NUMBER OF PAGES
69

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction

Estrogen is capable of driving cell cycle progression of hormone responsive breast cancers via high levels of estrogen receptor (ER). Historically, the primary mode of estrogen action has been considered to be through the transcriptional activation of genes containing estrogen response elements (EREs), including the immediate early genes c-myc and c-fos. However, recent reports suggest that estrogen also induces mitogen-activated protein kinase cascade (MAPK) activity in an ER-dependent manner suggesting that mitogenesis may be mediated through this cytoplasmic signaling cascade. The studies funded by this grant have focused on the role of several different cytoplasmic signaling cascades in estrogen-stimulated mitogenesis. First, specific small molecule inhibitors of MAPK (PD098059 and U0126), phosphotidylinositol 3-kinase (PI3-K) (LY294002 and wortmannin), protein kinase A (H-89), and protein kinase C (bisindolylmaleimide I) were used to determine the influence of these cascades on estrogen-mediated mitogenesis. Compounds capable of decreasing the fraction of cells entering DNA synthesis after treatment with estrogen were further studied to determine the nature and timing of their cell cycle block. Next, estrogen's ability to activate two kinase cascades essential to realize its mitogenic potential was characterized. Interestingly, neither of these kinase cascades was activated over basal levels in response to estrogen treatment. Since several publications have demonstrated a minor and transient activation of MAPK following estrogen treatment, a thorough examination of various conditions, including hormone concentration, serum concentration, cell density, and autocrine effects ensued. Supporting the role of cytoplasmic signaling cascades in estrogen-induced proliferation, Castoria *et al.* demonstrated that estrogen can drive cell cycle progression in the absence of ER-mediated transcription (Castoria et al., 1999). Therefore, minimal hormone concentrations required to induce mitogenesis or stimulate transcription were identified in a further attempt to separate these estrogenic actions. Finally, these analyses were validated in additional cell culture cell lines to ensure that the original observations were not specific to the MCF-7 line. Cumulatively, these studies indicate that estrogen initiates mitogenesis by inducing the transcription of immediate early genes, but cytoplasmic signaling pathways play an important role in the control of subsequent events in the cell cycle.

Body

Section I

Introduction

As clearly delineated in my two previous annual reports, the original Statement of Work has been dramatically altered to focus not on the breast cancer susceptibility gene BRCA2, but rather on the ability of estrogen to drive cell cycle progression of breast tissue. Specifically, the studies performed through the funding provided by this grant have attempted to further characterize the molecular mechanisms essential in estrogen-induced mitogenesis.

17β-estradiol (E_2), the most prevalent estrogen produced by the ovaries, can act as an effective mitogen in cells that express the ER both in cell culture and *in vivo* (reviewed in (Musgrove and Sutherland, 1994). Ligand-bound ER can interact with DNA at EREs, resulting in modulation of transcriptional activity. Anti-estrogen treatment of cells that depend upon estrogen for their growth blocks this transcriptional activation and results in a G0/G1cell cycle arrest (Musgrove et al., 1989). A number of genes implicated in cell cycle progression contain putative EREs including the immediate early genes, c-myc and c-fos (Dubik and Shiu, 1992; Weisz and Rosales, 1990). Therefore, it has been postulated that the mechanism by which E_2 mediates mitogenesis is via transcriptional activation of these and other key cell cycle regulatory genes.

After immediate early gene synthesis, the cell cycle converges on the expression and activity of the cyclins and cyclin dependent kinases (cdk), particularly the D-type cyclins. Increased expression of cyclin D1 results from E_2 treatment of growth-arrested MCF-7 cells (Prall et al., 1997). Concurrently, the activity of cyclin D1 binding partners, cdk4 and cdk6, increases (Matsushime et al., 1994; Meyerson and Harlow, 1994). Formation of active cyclin D1-cdk4 and cyclin D1-cdk6 holoenzymes serves two functions: (a) redistribution of cdk inhibitors (such as $p27^{Kip1}$ and $p21^{Cip1}$) away from cyclin E, facilitating the formation of active cyclin E-cdk2 complexes; and (b) phosphorylation of the Rb protein, which is also phosphorylated by cyclin E-cdk2 (Connell-Crowley et al., 1997; Polyak et al., 1994; Reynisdottir et al., 1995).

The subcellular localization of the ER and its role as a transcription factor may lead to the assumption that E_2 action occurs strictly in the nucleus. However, several recent reports have shown that elements of cytoplasmic signaling cascades may also be E_2-responsive. In cells expressing ER, MAPK family members Erk1 and Erk2 are activated within 5 minutes of E_2 treatment (Di Domenico et al., 1996; Endoh et al., 1997; Migliaccio et al., 1996; Singh et al., 1999). Furthermore, cell membrane-impermeable E_2 (E_2-conjugated to bovine serum albumin) stimulates MAPK activity (Watters et al., 1997), indicating that MAPK activation may be the result of E_2 interacting with a cell surface protein. Activation of MAPK raises the possibility that some or all E_2's mitogenic activity may be mediated through this pathway.

This theory was also supported by a study demonstrating that a MAPK inhibitor (PD098059) prevented E_2-induced proliferation in cardiac fibroblasts (Lee and Eghbali-Webb, 1998). E_2 has also been shown to induce mitogenesis in NIH-3T3 cells transiently transfected with transcriptionally inactive ER, presumably through the activation of MAPK (Castoria et al., 1999). Combined, these studies suggest that MAPK activation may be necessary and sufficient for E_2-stimulated cell cycle progression.

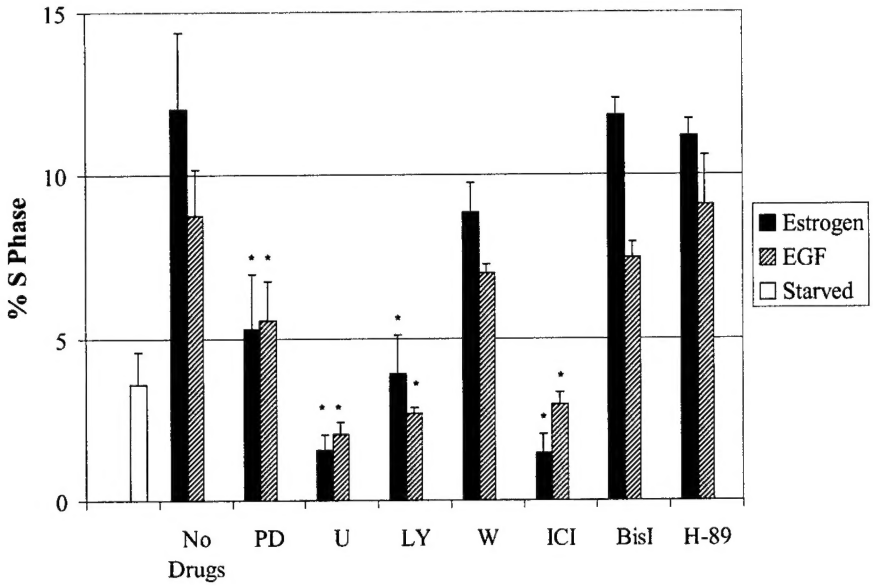
E_2 and the ER may also interact with other cytoplasmic signaling cascades. In endometrial fibroblasts as well as selected malignant cell lines, E_2 stimulates the activity of protein kinase C (PKC) (Fujimoto et al., 1996). In MCF-7 cells, synthesis of phosphatidylinositol and activation of phosphatidylinositol kinases can occur in response to E_2 treatment (Etindi et al., 1992). The ER itself can also act as a substrate for protein kinase A (PKA); serine-236 can be phosphorylated *in vitro* by PKA, which may be important in receptor dimerization (Chen et al., 1999). These reports suggest that cytoplasmic factors may be important intermediates in E_2 action.

In previous work, our laboratory found that MCF-7 cells grown in medium alone (no serum or added growth factors) proliferate in response to E_2 (Vaughn et al., 1996). This system provides a model for studying the mitogenic effects of E_2 in the absence of other exogenously added stimulatory molecules. Using this model system, I tested a series of pharmacological compounds (mechanisms of action detailed in Appendix A) that specifically inhibit signaling intermediates that have been implicated in estrogenic action. My results show that MAPK and phosphatidylinositol 3-kinase (PI3-K) activity, but not PKA or PKC activity, are required for cell cycle progression in response to E_2 stimulation. The G1 arrest generated by MAPK and PI3-K inhibitors occurs after the expression of immediate early genes and prior to the accumulation of cyclin D1.

Cytoplasmic Signaling Inhibitors Prevent E_2-Induced Mitogenesis

The breast cancer epithelial cell line MCF-7 expresses high levels of ER. When these cells are serum-starved in phenol red-free media for 24 hours, the largest fraction of cells accumulates and arrests early in the G1 stage of the cell cycle. Treatment of starved cells with E_2 alone stimulates cell cycle progression and results in a 3- to 4-fold increase in the number of cells undergoing DNA synthesis, a rate that is comparable or greater than the mitogenic effect of EGF (Figure 1A).

A.



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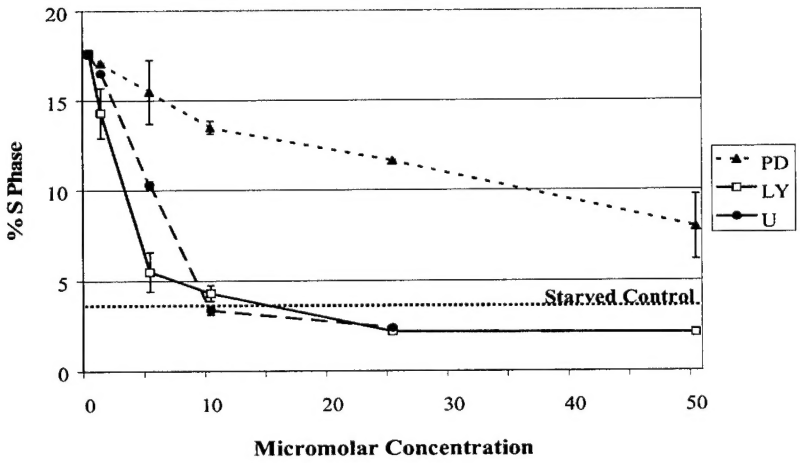


Figure 1. Effect of cytoplasmic signaling inhibitors on E_2-induced mitogenesis. A) Percentage of MCF-7 cells in S phase after treatment with 2 X 10^{-8}M E_2 or 10 ng/ml EGF for 24 hours in the presence or absence (No Drugs) of 50 μM PD098059 (PD), 25 μM U0126 (U), 5 μM LY294002 (LY), 100 nM wortmannin (W), 2 μM ICI 182,780 (ICI), 750 nM bisindolylmaleimide I (BisI), or 500 nM H-89. Compounds such as ICI 182,780, which inhibit MCF-7 cells by generating a potent cell cycle block, will exhibit lower rates of DNA synthesis than the asynchronous serum-starved control. The data represent an average of results obtained from three experiments, each performed in triplicate. * denotes statistically significant inhibition of estrogen-induced mitogenesis at $p \leq 0.05$. *Bars,* SD. B) E_2-stimulated MCF-7 cells were treated with 1, 5, 10, 25, or 50 μM PD098059 (PD), LY294002 (LY), or U0126 (U) for 24 hours and the rate of DNA synthesis was assessed flow cytometrically after the cells were stained with propidium iodide. The data represent an average of results obtained from three experiments, each performed in triplicate. *Bars,* SD.

Several signaling cascades have been implicated in E_2 function. To assess the importance of these pathways on E_2-induced mitogenesis, I examined changes in the rate of DNA synthesis as a result of exposure to specific pharmacological inhibitors. Serum starved cells were stimulated with 2 X 10^{-8}M E_2 in the presence of inhibitors of MEK, the upstream activator of Erk1/Erk2 (50 μM PD098059 or 25 μM U0126), PI3-K (5 μM LY294002 or 100 nM wortmannin), PKA (500 nM H-89), PKC (750 nM bisindolylmaleimide I), or with an ER antagonist (2 μM ICI 182,780). These concentrations were chosen based upon published values demonstrated to inhibit each of the kinases effectively without causing non-specific toxicity (Alessi et al., 1995; Chijiwa et al., 1990; Toullec et al., 1991; Vlahos et al., 1994). Cell cycle distribution was measured by flow cytometry after 24 hours of treatment (Figure 1). The anti-estrogen, as expected, effectively blocked E_2-induced DNA synthesis, reducing the percentage of cells in S phase to below the levels in starved cells. Neither the PKA nor PKC inhibitors (H-89 and BisI) had any significant effect. However, the MEK1 inhibitor, PD098059 (PD), reduced the rate of DNA synthesis by greater than 50%, whereas the MEK1/MEK2 inhibitor, U0126 (U), decreased the rate of proliferation to below the level observed in starved cells. Inhibition of the PI3-K cascade with LY294002 (LY) was almost as effective as the ICI anti-estrogen in blocking E_2-induced S phase. However, wortmannin (W) was not as efficacious an inhibitor. These data suggested that the activities of at least two cytoplasmic signaling molecules (MEK1 and PI3-K) are critical for the mitogenic effect of E_2.

To compare the effects of these inhibitors on another ligand that is mitogenic in MCF-7 cells, we repeated the experiments on this breast cancer cell line stimulated with EGF (Imai et al., 1982). As previously reported (Wosikowski et al., 1993), the anti-estrogen ICI 182,780 inhibited EGF-induced mitogenesis. Furthermore, EGF-stimulated DNA synthesis was inhibited by the same compounds to virtually the same degree as was seen with E_2 treatment (Figure 1A).

In order to determine the efficacy of these drugs in inhibiting E_2-induced proliferation, we measured S phase progression in the presence of a range of inhibitor concentrations (1-50 μM). At the lowest concentration for each drug, minimal effects, if any, were observed (Figure 1B). However, in the presence of 5 μM LY294002, the percentage of cells in S phase was maintained at or near basal levels, a finding that is supported by the published IC_{50} (1.4 μM) for abolishment of PI3-K activity (Vlahos et al., 1994). Similar kinetics of inhibition were noted with the MEK1/MEK2 inhibitor, U0126. PD098059 only weakly inhibits the activation of MEK2 and has a much greater IC_{50} for the inhibition of MEK1 as compared to U0126 (Favata et al., 1998). Therefore, it was not surprising to note that PD098059 did not have as pronounced an inhibitory effect on E_2-induced mitogenesis.

Toxicity and Reversibility of PI3-K and MAPK Inhibitor Molecules

The toxicity and reversibility of the pharmacological inhibitors of MAPK, PI3-K, or PKA were examined next. Cell viability was measured by propidium iodide dye exclusion assay after 48 hours of drug treatment (Table 1). U0126 and LY294002 had little or no effect on cellular viability with concentrations less than 50 μM. PD098059 had no effect on viability up to 10 μM; however, a 50 μM dose decreased viability by roughly 7%. Inhibition of PKA (H-89) had no effect on cell viability with doses up to 1 μM. Additionally, there was no increase in the apoptotic fraction in the presence of these drugs as measured by flow cytometry (data not shown).

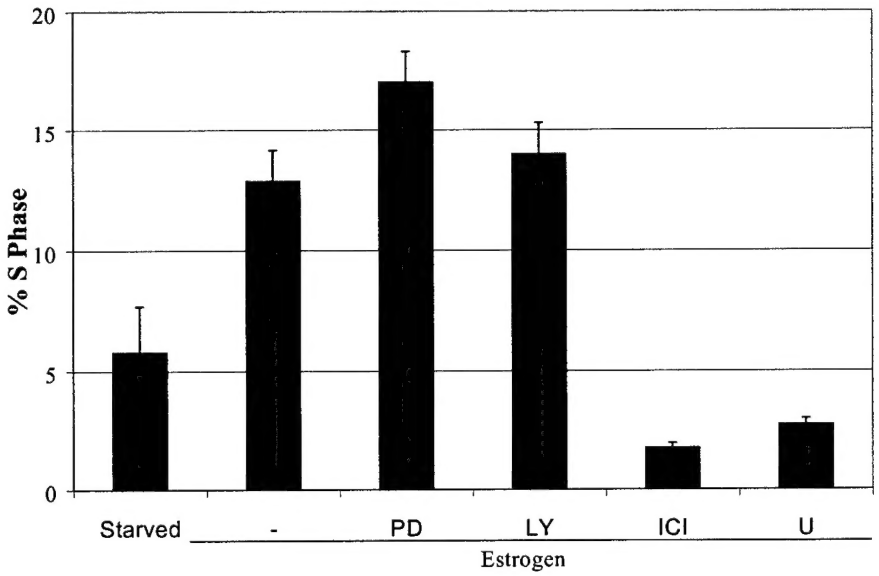
After demonstrating that these compounds were not exerting cytotoxic effects on MCF-7 cells, I wanted to ensure that the drugs were not displaying cytostatic effects by irreversibly affecting the cell cycle machinery. Therefore, the reversibility of the growth inhibitory effects of each drug was measured by a washout experiment. MCF-7 cells were treated with the small molecule inhibitors for 24 hours in the absence of any mitogen. Cultures were then washed three times with PBS and stimulated with E_2 (Figure 2). Cell cycle analysis showed that cells treated with 5 μM LY294002 and 50 μM PD098059 could respond mitogenically to E_2 stimulation after the inhibitors had been removed (Figure 2A). However,

inhibition by 25 μM U0126 and 2 μM ICI 182,780 could not be reversed in this time frame or after 48 hours of hormone stimulation (Figure 3A and data not shown). Further examination using variable doses of both compounds revealed that 10 μM U0126, which still is capable of maximal inhibition of E_2-induced proliferation, is reversible (Figure 2B). Removal of 0.2 μM ICI 182,780 enables subsequent cell cycle progression; however, this concentration of anti-estrogen is not as potent at inhibiting E_2-induced mitogenesis as the 2 μM concentration routinely used in these experiments. These findings demonstrate that PD098059, LY294002, and U0126 can be reversible inhibitors at concentrations that generate a potent cell cycle block. However, concentrations of ICI 182,780 that induce maximal cell cycle arrest are not reversible. Since, ICI 182,780 induces ER degradation, a long-lasting effect on ER responsiveness was not surprising (Parker et al., 1993).

Condition	*Dose*	*Viability*
Log		94.6%
Starved	Starved	93.1%
U0126	5 μM	93.6%
	10 μM	93.0%
	25 μM	96.4%
	50 μM	95.2%
	100 μM	86.6%
PD098059	5 μM	94.2%
	10 μM	93.9%
	25 μM	88.8%
	50 μM	85.9%
LY294002	5 μM	92.4%
	10 μM	94.0%
	25 μM	94.7%
	50 μM	92.4%
	100 μM	88.8%
H-89	500 nM	93.6%
	1 μM	93.7%
	10 μM	86.0%

Table 1. MCF-7 cell viability in the presence of variable concentrations of pharmacological inhibitors. MCF-7 cells were treated with the variable doses of U0126, PD098059, LY294002, or H-89 for 48 hours. After drug treatment, adherent cells as well as the suspended cells were harvested and washed. Cell pellets were resuspended in complete medium containing propidium iodide. The percentage of cells that excluded the dye (viable) was assessed by flow cytometric analysis.

A.



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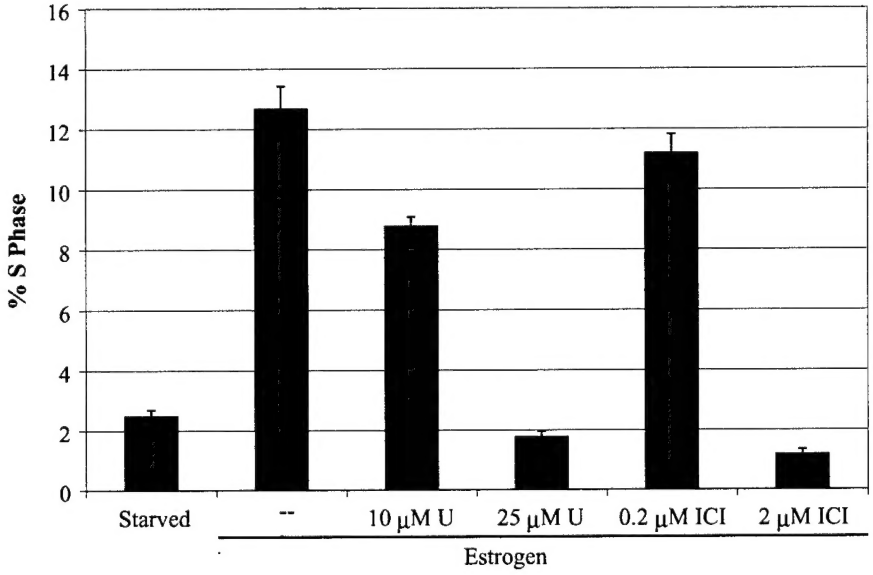


Figure 2. Reversibility of cytoplasmic signaling inhibitors. A) Serum-starved MCF-7 cells were treated with 50 μM PD098059 (PD), 5 μM LY294002 (LY), 2 μM ICI 182,780 (ICI), or 25 μM U0126 (U) for 24 hours. Cells were then washed three times with PBS prior to stimulation with 2 X 10^{-8}M E_2 in the absence of any inhibitory compounds for 24 hours. The rate of DNA synthesis was then measured by propidium iodide staining and analysis by FACS. B) The reversibility of 10 and 25 μM U0126 (U) as well as 0.2 and 2 μM ICI 182,780 (ICI) was assessed using the identical technique detailed in A.

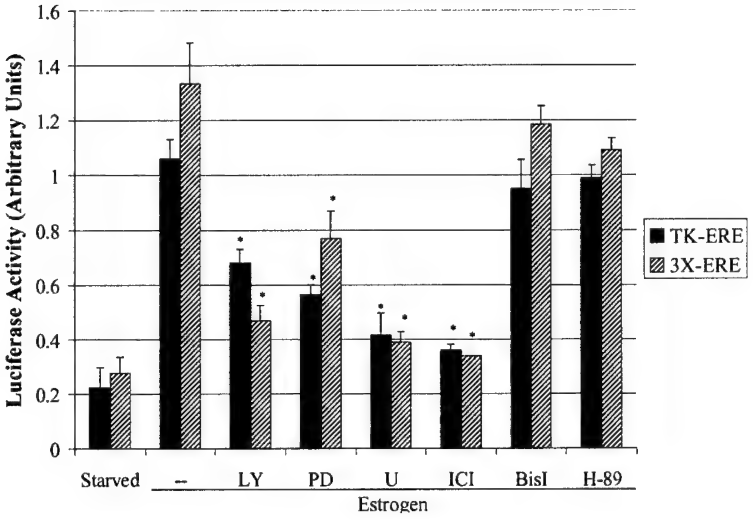
Inhibitor Effects on ER-Mediated Transcription

The most well understood mode of E_2 action is the transcriptional activation of genes containing estrogen response elements (EREs). Among the genes containing putative EREs are several key cell cycle regulatory genes, including the immediate early genes c-myc and c-fos (Dubik and Shiu, 1992; Weisz and Rosales, 1990). If the PI3-K and MAPK inhibitors affected transcriptional regulation by ER, they could stop cell cycle progression by preventing the induction of these genes. To determine whether the effects of signaling inhibitors on E_2-stimulated mitogenesis were attributable to interference with ER-mediated transcriptional activity, MCF-7 cells were transfected with a luciferase gene under the control of either a naturally occurring (vitellogenin, TK-ERE) or artificial (3X-ERE) E_2-responsive promoter. Cells were stimulated for 24 hours with E_2 prior to harvesting cell extracts and measuring luciferase activity. In the absence of drugs, E_2 induced a 5- to 6-fold increase in luciferase activity as opposed to the near basal levels seen in the presence of the ER antagonist, ICI 182,780 (Figure 3A). Blocking PKA (H-89) or PKC (BisI) activity had no impact on ER-mediated transcription, whereas the MEK1/2 inhibitor U0126 was as effective as ICI 182,780 at preventing ER-mediated transcription. Transcription declined approximately 50% in the presence of the MEK1 inhibitor PD098059 or the PI3-K inhibitor LY294002.

To examine the impact of cytoplasmic signaling cascades on earlier transcriptional events, this experiment was repeated with cell extracts harvested at shorter intervals. Samples taken after 6 or 9 hours of E_2 stimulation displayed the same kinetics of induction and inhibition as the 24 hour time point (data not shown). Attempts at detecting even earlier effects (2 hours) did not yield interpretable results since E_2 alone failed to induce detectable luciferase activity. This is most likely due to insufficient time for the processes of transcription as well as translation to produce functional luciferase protein. Since this assay requires protein synthesis in order for a functional read-out of ER-mediated transcriptional events, one potential explanation for these results is that the inhibitors of MAPK and PI3-K activity are effecting the translation of luciferase transcripts. Alternatively, these studies may indicate that inhibition of MAPK and PI3-K activity directly results in decreased ER-mediated transcription, though it cannot be discerned whether this is an immediate or delayed effect.

I examined the specificity of these inhibitory effects on ER-mediated transcription by studying the impact of the inhibitors on the transcriptional activity of another steroid hormone, progesterone. MCF-7 cells were transiently transfected with a human PR-B expression vector and a luciferase gene with a progesterone-responsive promoter. Addition of 10^{-7}M progesterone for 24 hours stimulated a 5-fold increase in luciferase activity, an activity that was not inhibited in the presence of LY294002 and ICI 182,780 (Figure 3B). Both MAPK inhibitors prevented maximal induction by approximately 50%. Therefore, these cytoplasmic inhibitors have pleiotropic effects on nuclear hormone mediated transcription.

A.



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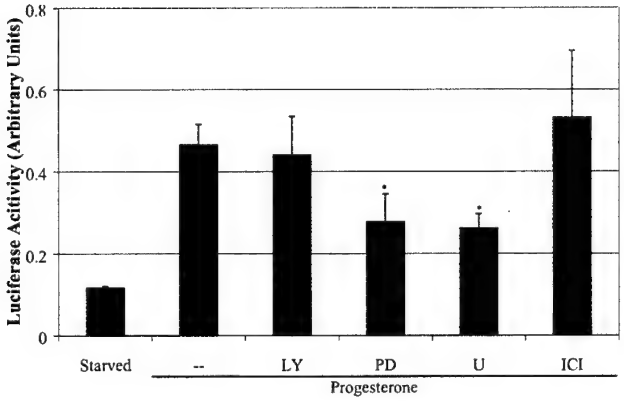


Figure 3. Effect of cytoplasmic inhibitors on ER-dependent transcription. A) MCF-7 cells were transfected with a luciferase construct containing either an artificial (3X-ERE) or a naturally occurring (TK-ERE) estrogen-responsive promoter. Samples were harvested after 2, 6, 9 or 24 hours of stimulation with 2 X 10^{-8}M E_2 in the presence or absence (--) of 5 μM LY294002 (LY), 50 μM PD098059 (PD), 25 μM U0126, 2 μM ICI 182,780 (ICI), 750 nM bisindolylmaleimide I (BisI), or 500 nM H-89. Cells were lysed and luciferase activity was assessed. The 2-hour interval was not sufficient to generate detectable levels of luciferase activity; however, the 6- and 9-hour time points displayed similar kinetics of induction and inhibition as the 24-hour results shown. Corrections for differences in protein concentration and transfection efficiencies were made using detected levels of a CMV-luciferase construct from a different species, and are given for three experiments performed in triplicate. * denotes statistically significant inhibition of ER-mediated transcription at $p \leq 0.05$. *Bars,* SD. B) MCF-7 cells were transfected with a human PR-B expression construct in addition to a progesterone-responsive luciferase construct. 24 hours after treatment with 10^{-7}M progesterone and inhibitory compounds, cells were lysed and luciferase activity for measured and corrected as detailed above. * denotes statistically significant inhibition of PR-mediated transcription at $p \leq 0.05$. *Bars,* SD.

The level of ER itself may be decreasing in response to the inhibitory compounds, which could account for the decline in transcriptional activity. Therefore, we measured the levels of ER by immunoblotting over a 24 hour treatment regimen (Figure 3-4). ER is known to decline in response to E_2 treatment (Read et al., 1989; Ree et al., 1989). A 3-fold reduction of ER protein was detected after 9 hours of E_2 stimulation. E_2 stimulation in the presence of LY294002 (LY), wortmannin (W), or PD098059 (PD) did not affect ER protein levels compared to E_2 alone. Furthermore, in the absence of E_2, these drugs caused no change in the steady-state levels of ER found in serum-starved cells. Consistent with published data, ICI 182,780 reduced ER levels in the presence or absence of steroid hormone (Parker et al., 1993). Unlike PD098059, treatment with the U0126 decreased ER levels with kinetics similar to those observed with ICI 182,780. Interestingly, progesterone receptor turnover is mediated by phosphorylation of serine-294 by MAPK, and inhibition of MAPK activity prevents ligand-dependent receptor down-regulation (Lange et al., 2000). The finding that PD098059 does not affect E_2-dependent receptor turnover, while U0126 increases the rate of ER downregulation indicates that the molecular mechanisms for receptor turnover are different for these two different nuclear receptors. However, the reduction of ER in the presence of U0126 and with ICI 182,780 is consistent with the dramatic inhibition of ER-mediated transcription at later time points.

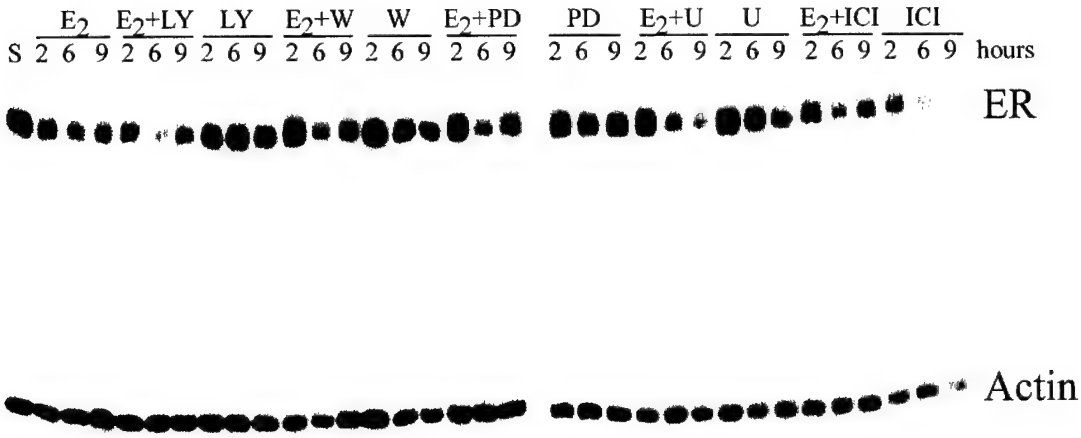


Figure 4. Effect of cytoplasmic inhibitors on ER expression levels. MCF-7 cells were serum-starved for 24 hours (S). Cells were then incubated with 5 μM LY294002 (LY), 100 nM wortmannin (W), 50 μM PD098059 (PD), 25 μM U0126 (U), or 2 μM ICI 182,780 in the presence or absence of 2 X 10^{-8}M E_2 for 2, 6, or 9 hours prior to lysis. Western analysis was utilized to detect levels of ER present in each condition. This blot was reprobed to quantitate actin levels in order to control for loading differences. Data are representative of six experiments.

Inhibitor Effects on E_2-Induced Gene Transcription

The kinase inhibitors did exert a suppressive effect on E_2-mediated transcription measured by the reporter gene assay. These assays were performed between 6 and 24 hours of drug treatment, long after the signaling cascades or the ER may transduce any immediate effects on the cell cycle. Therefore, decreased ER transcription in these assays may have been due to secondary effects of the arrest rather than a direct effect of kinase inhibitors.

Expression of the immediate early genes occurs rapidly after E_2 treatment; therefore, the expression of these genes was measured directly by Northern blotting (Figure 5). Maximal expression of the two putative E_2-responsive immediate early genes, c-myc and c-fos, was observed within one hour of E_2 treatment. None of the signal transduction inhibitors (bisindolylmaleimide I, H-89, LY294002, PD098059) had an effect on the kinetics or magnitude of E_2-induced myc and fos expression (Figure 3-5 and data not shown). E_2 induction of myc and fos protein was also not inhibited by these compounds as measured by Western analysis (data not shown). ICI 182,780 completely inhibited the induction of both c-myc and c-fos mRNA (and protein) as expected (Figure 5 and data not shown). Despite decreasing the steady-state levels of ER, U0126 had no effect on the induction of myc and fos in response to E_2 treatment. Therefore, while the compounds did effect E_2 transcription measured in the transient assays, they had no effect on immediate early gene synthesis. This may be attributable to the different "read-outs" utilized in assaying ER-mediated transcription. For immediate early genes, transcription is assessed at the RNA level by Northern blotting, whereas the luciferase assay is measured after protein synthesis has also occurred. Therefore, one potential explanation for these apparently discrepant results is that MAPK activity is involved in the process of protein synthesis. Furthermore, inhibition of ER-mediated transcription in the reporter gene assay at later times does not correlate with the immediate effects of E_2-induced transcription of endogenous genes. Cumulatively, these results suggest that induction of myc and fos is directly dependent upon the hormone receptor's nuclear action.

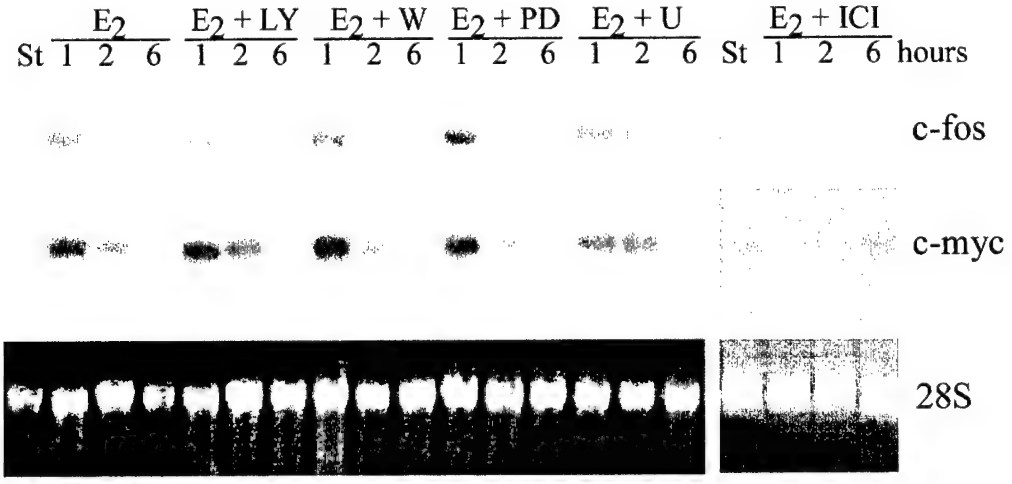


Figure 5. Effect of cytoplasmic signaling inhibitors on E_2-induced immediate early gene RNA levels. Representative Northern blot analysis of c-myc and c-fos RNA expression in MCF-7 cells treated with E_2 in the presence or absence of 5 μM LY294002 (LY), 100 mM wortmannin (W), 50 μM PD098059 (PD), 25 μM U0126 (U), or 2 μM ICI 182,780 (ICI) (from a separate Northern blot) for 1, 2, or 6 hours. The starved lane displays the amount of c-myc or c-fos RNA present at time 0. Levels of the 28S ribosomal subunit were used as an internal control to correct for loading inconsistencies.

Timing of the Mitogenic Block

It is readily understandable how the ER antagonist ICI 182,780 blocks cell cycle progression via inhibition of immediate early gene expression (Cicatiello et al., 1993; Hyder et al., 1997). However, the cytoplasmic signaling inhibitors did not prevent the immediate early genes from being induced. This finding raises the question of where in the cell cycle the block is imposed. To investigate the timing of this inhibition, E_2 was added to serum-starved MCF-7 cells at time 0. Inhibitory compounds (PD098059, U0126, LY294002, or ICI 182,780) were added at varying times after the mitogenic stimulus (Figure 6). At 24 hours, all cultures were harvested and the percentage of cells in S phase was determined. Our laboratory had previously demonstrated that starved MCF-7 cells begin to enter S phase approximately 12 hours after the addition of E_2 with peak S phase values reached at 24 hours (Vaughn et al., 1996). Effective inhibition of cell cycle progression was observed when ICI 182,780, LY294002, or U0126 was added up to 6 hours after exposure to E_2. By 12 hours, none of these compounds were effective inhibitors. In parallel experiments using the MEK1 inhibitor (PD098059), we observed a gradual increase in S phase progression after delayed addition of the compound. While the demarcation in PD098059's inhibitory activity was not as clearly defined, the profiles from PD098059, U0126, and LY294002 suggested that inhibition of events early in G1 are important for the activity of these drugs in preventing S phase progression.

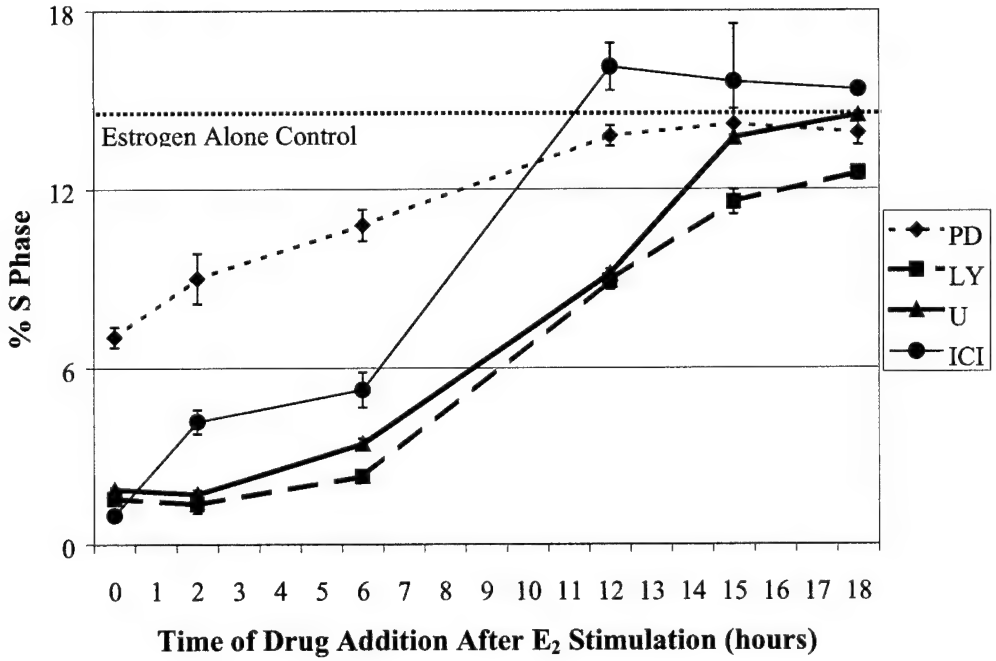


Figure 6. Mitogenic Effect of Adding Cytoplasmic Signaling Inhibitors after E_2–Stimulation. Starved cultures of MCF-7 cells were treated with 2 X 10^{-8}M E_2 at time 0 and then treated with either 50μM PD098059 (PD), 5μM U0126 (U), 5μM LY294002 (LY), or 2μM ICI 182,780 at 2, 6, 12, 15, or 18 hours. At 24 hours, cell cultures were harvested and the fraction of cells in S phase was measured by flow cytometry.

Inhibitor Effects on E_2-Induced Cyclin D1 Expression and Rb Phosphorylation

Increased cyclin D1 expression is considered to be a delayed-early event in cell cycle progression that occurs after immediate early genes are induced. Accumulation of cyclin D1 protein after E_2 stimulation is a critical event in G_1 progression (Planas-Silva and Weinberg, 1997). In addition, overexpression of cyclin D1 can reverse the cell cycle (Watts et al., 1995), and the MAPK inhibitor U0126 both prevented cyclin D1 induction and activation of cdk2, and led to decreased levels of hyperphosphorylated Rb protein. These findings are consistent with the effects of ICI 182,780 and U0126 on mitogenesis. Of the PI3-K inhibitors, wortmannin did not prevent the accumulation of cyclin D1 protein to the same extent as LY294002, a finding that is consistent with the relative S-phase inhibition observed with these compounds. The MEK1 inhibitor PD098059 also inhibited cyclin D1 levels; however, the effect appeared to be more transitory as cyclin D1 began to accumulate at later time points (Figure 7A and data not shown). This was also reflected by the incomplete inhibition of cdk2 activation, Rb hyperphosphorylation, and cell cycle progression. From these experiments, we place the critical anti-mitogenic effects of the PI3-K and MAPK inhibitors at or before the level of cyclin D1 accumulation.

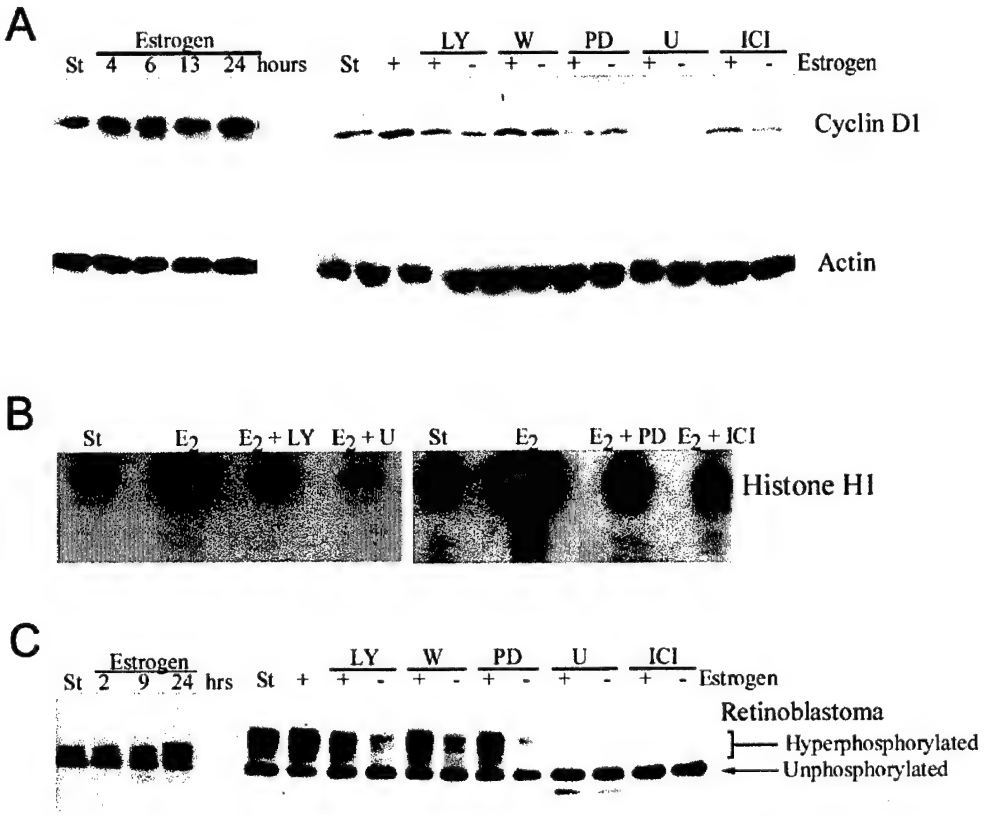


Figure 7. Effects of cytoplasmic signaling inhibitors on cyclin D1 expression, cdk2 activity, and Rb phosphorylation. Cells were incubated with 5 μM LY294002 (LY), 100 nM wortmannin (W), 50 μM PD098059 (PD), 25 μM U0126 (U), or 2 μM ICI 182,780 (ICI) in the presence or absence of 2 X 10^{-8}M E_2. A) After 6 hours, the cells were lysed and relative amounts of cyclin D1 and actin were detected by immunoblotting. B) After 24 hours of treatment, cells were lysed and cdk2 was immunoprecipitated; the activation status of this kinase was assayed by determining its ability to phosphorylate histone H1. C) Rb phosphorylation status was assessed using Western analysis on cell lysates that were extracted after 24 hours of stimulation.

Discussion

Previous experiments on E_2-induced growth has led to several conclusions: (a) the response depends upon the presence of functional ER, (b) immediate early genes are transcriptionally activated, and (c) cyclin D1 is an important intermediate in the proliferative response (reviewed in (Prall et al., 1998b). If one assumes that the primary mode of E_2 action is via its receptor acting as a ligand-inducible transcription factor, mitogenesis could be explained by the direct transcriptional activation of immediate early genes that contain functional EREs. The recent reports of E_2 activation of cytoplasmic signaling components, particularly the MAPK cascade, complicate this relatively simple paradigm (Chen et al., 1999; Di Domenico et al., 1996; Endoh et al., 1997; Etindi et al., 1992; Fujimoto et al., 1996; Migliaccio et al., 1996; Ruzycky, 1996; Watters et al., 1997). It is in this context that the current studies were performed.

I found that the ability of MCF-7 cells to enter S-phase in response to E_2 was prevented or reduced by the disruption of the PI3-K and MAPK cascades by specific inhibitors (PD098059 which inhibits MEK1, U0126 which inhibits MEK1 and MEK2, and LY294002 and wortmannin which inhibit PI3-K). These inhibitors arrested cells in G1 and did not induce any significant cytotoxicity. They also demonstrated variable effects on E_2-mediated transcription when assayed using reporter constructs; however, the immediate early genes c-myc and c-fos were both induced in the presence of these drugs. Both the PI3-K and MAPK inhibitors prevented cyclin D1 accumulation, which correlated with an absence of cdk2 activity and Rb phosphorylation. Therefore, the inhibition of E_2-mediated mitogenesis by these drugs is likely attributable to this repression of cyclin D1 expression, cdk2 activation, and Rb phosphorylation.

This study raises several issues regarding the mechanism of E_2 action and mitogenesis in general. MAPK and PI3-K inhibitors were shown to inhibit ER-mediated transcription, as assayed using a gene reporter system. However, these inhibitors did not prevent the expression of immediate early genes in response to E_2. At least one plausible explanation can reconcile this apparent discrepancy. Maximal expression of c-myc and c-fos occurred after 1 hour of stimulation, whereas luciferase activity required 6 hours for a measurable response. Therefore, effects on ER transcription may not be manifest until after the rapid immediate early response. How critical this delayed inhibition of ER activity is in mediating the growth arrest remains to be determined.

E_2 stimulates the production and secretion of growth factors that can function in an autocrine fashion (Cullen et al., 1989). For example, protein levels of fibroblast growth factor-1 (FGF-1) increase in response to E_2 treatment in MCF-7 cells and may act synergistically with the hormone to generate a greater proliferative response (El Yazidi et al., 1998; Zhang et al., 1997). bFGF is also up-regulated in response to E_2 stimulation of human endothelial cells and is required for the MAPK activation that is observed 3 hours after exposure to E_2 (Kim-Schulze et al., 1998). Continuous incubation in the presence of MEK inhibitors would prevent the activation of MAPK by growth factors acting in an autocrine loop. Therefore, the cell cycle block observed with PD098059 and U0126 may be a result of inhibiting autocrine cascades necessary to realize the mitogenic potential of E_2. My finding that an anti-proliferative response can still be achieved when adding the inhibitory drugs up to 6 hours after hormone stimulation further substantiates this claim.

The results of the experiments detailed thus far strongly suggest that cyclin D1 induction is a key intermediate in E_2-induced proliferation. Several reports have focused on the role of cyclin D1 activation in E_2-mediated mitogenesis. Planas-Silva and Weinberg demonstrated that E_2 relieves a cell cycle block in tamoxifen-arrested MCF-7 cells by increasing levels of cyclin D1 protein, which causes the redistribution of $p21^{cip1/waf1}$ away from the cyclin E/cdk2 complexes, allowing Rb phosphorylation and cell cycle progression (Planas-Silva and Weinberg, 1997). Prall *et al.* derived MCF-7 cell lines with

inducible expression of c-myc or cyclin D1 (Prall et al., 1998a). Cell cycle arrest of these lines by the estrogen antagonist, ICI 182,780, was reversed by expression of either myc or cyclin D1. In both cases, loss of the cdk inhibitor p21 from cyclin E/cdk2 complexes was noted. Interestingly, c-myc overexpression did not result in elevated cyclin D1 levels, and when cyclin D1 was induced, myc levels remained constant, implicating the existence of multiple pathways to remove p21. My data indicates that c-myc induction is not sufficient to allow E_2-mediated cell cycle progression in the face of diminished cyclin D1 expression.

The molecular mechanism by which the PI3-K cascade functions in cyclin D1 accumulation has begun to be characterized as a posttranscriptional phenomenon. In the absence of mitogen, cyclin D1 is targeted for ubiquitin-dependent degradation due to the presence of a phosphate group at threonine 286, a substrate for glycogen synthase kinase 3-β (GSK-3β) (Diehl et al., 1998; Diehl et al., 1997). Inactivation of GSK-3β occurs as a result of PKB activation, a downstream target of the PI3-K cascade (Watts et al., 1995) and reviewed in (Coffer et al., 1998). Thus, the cell cycle block induced by LY294002 in the presence of E_2 may be attributable to the inability of GSK3-β to be inactivated by PKB, thereby preventing the accumulation of cyclin D1.

The role of MAPK in cyclin D1 accumulation is less well defined. Typically, activation of MAPK results in nuclear translocation and activation of immediate early gene expression. Several studies, however, do suggest a more direct effect. Using a Chinese hamster fibroblast cell line, CCL39, Lavoie *et al.* demonstrated that not only was MAPK activation required for expression of endogenous cyclin D1, but also sufficient for full induction of this protein in the absence of growth factors (Lavoie et al., 1996). One theory to explain this phenomenon is that activated MAPK phosphorylates PHAS-1, which in turn impacts mRNA cap recognition and translation via eIF-4E (Lin et al., 1994). Therefore, inhibition of these cascades may prevent cyclin D1 accumulation at the level of mRNA utilization.

The data presented here reveal that inhibition of the MAPK and PI3-K cytoplasmic signaling cascades prevents E_2-induced mitogenesis. The finding that immediate early gene expression is unaffected by the inhibitory compounds suggests that ER-mediated transcriptional events are still required in this process. This conclusion underscores the importance of determining the downstream molecular events required for the accumulation of cyclin D1 protein that result from MAPK activation. Also of interest is whether E_2 directly activates MAPK and PI3-K or indirectly through the expression of growth factors which function in autocrine loops.

Section II

Introduction

As detailed previously, the classic model explaining the molecular mechanism by which E_2 induces proliferation revolves around the activity of ER as a ligand-dependent transcription factor. The ability of ER to coordinate expression of genes essential for mitogenesis is thought to be the driving force behind cell cycle progression.

Recent findings have called this model into question. Several studies have demonstrated that E_2 can activate several different cytoplasmic signaling cascades. In MCF-7 cells, E_2 increases synthesis of phosphatidylinositol and activates phosphatidylinositol kinases (Etindi et al., 1992). E_2 activation of the MAPKs Erk1 and Erk2 occurs in a variety of cell types, including breast epithelium (reviewed in (Collins and Webb, 1999). Support for the regulation of MAPK by E_2 was demonstrated *in vivo* using a rat mammary carcinoma model. In this system, oophorectomy diminished the quantity of total and active MAPK present in the cancer cells (Maemura et al., 1999). Administration of E_2 increased both of these levels, suggesting that MAPK expression and activation can be regulated by E_2.

Activation of the MAPK cascade raises the possibility that some, or all, of the proliferative effect of E_2 may be mediated through this established mitogenic pathway. This theory was supported by the

finding that PD098059, an inhibitor of MAPK activation, prevents E_2-induced mitogenesis in cardiac fibroblasts (Lee and Eghbali-Webb, 1998), as well as in MCF-7 cells as detailed in Section I. Furthermore, E_2 was shown to stimulate cell cycle progression in NIH-3T3 cells transiently transfected with an ER mutant that had no transcriptional activity, presumably by activating MAPK (Castoria et al., 1999). Cumulatively, these studies indicate an involvement of the MAPK cascade in the proliferative response to E_2 stimulation.

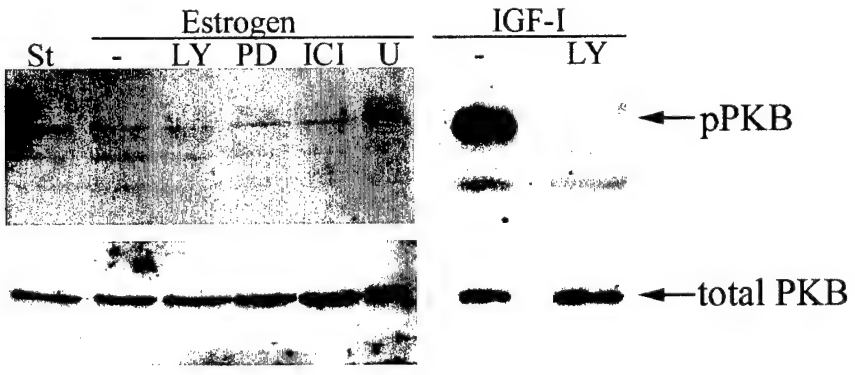
In the studies presented in this section, I further explore the relationship between signal transduction pathways and E_2-induced mitogenesis. To accomplish this goal, I performed experiments assessing the activation of both MAPK as well as PKB, a downstream target of PI3-K, in response to E_2 stimulation. I determined that no immediate activation of either kinase could be detected in the presence of mitogenic concentrations of E_2. Furthermore, delayed activation of the MAPK was not observed, either from the direct effect of E_2 or from the indirect effect of an autocrine loop. Inhibition of mitogenic growth factors by neutralizing antibodies failed to inhibit the proliferative effects of E_2. In addition, we demonstrate that E_2-induced transcription cannot be uncoupled from E_2-stimulated proliferation. Combined with data presented in Chapter 3, these findings indicate that E_2-induced cell cycle progression requires basal levels of active Erk1 and Erk2 rather than further activation of the MAPK cascade.

Effect of E_2 on the Activation of the PI3-K Cascade

PKB is a primary downstream target of PI3-K activation and has been implicated in cyclin D1 protein stabilization (Muise-Helmericks et al., 1998). Contrary to recently published data, treatment with E_2 had little, if any, effect on phosphorylated (active) PKB levels compared to stimulation with 30 ng/ml insulin-like growth factor-I (IGF-I), a known activator of PI3-K in MCF-7 cells (Figure 8A) (Dufourny et al., 1997). Neither PD098059, U0126, nor ICI 182,780 affected the phosphorylation of PKB; however, the PI3-K inhibitory drug, LY294002, was a potent inhibitor of PKB activation in response to IGF.

The effect of LY294002 on the basal levels of phosphorylated PKB (pPKB) was also investigated. Only at the highest concentration (50 μM) was pPKB significantly inhibited relative to starved or E_2-treated cells (Figure 8B). However, near-maximal inhibition of S-phase was achieved using 5 μM (Figure 1B). As measured by phosphorylation of PKB, inhibition of the basal activity of PI3-K does not appear to correlate with suppression of E_2-induced growth.

A.



B.

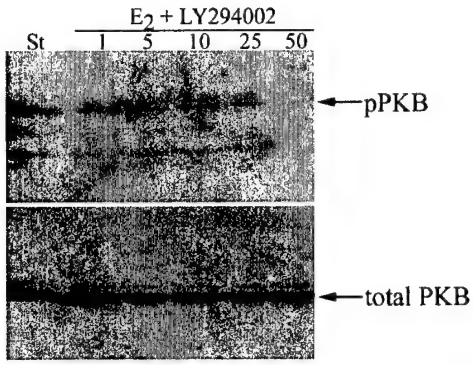


Figure 8. Effect of E_2-stimulation on PKB phosphorylation status. A) Phosphorylation of PKB was determined by Western analysis using a phospho-specific antibody on MCF-7 cell extracts from cells stimulated with 30 ng/ml IGF-I in the presence or absence of 5 μM LY294002 (LY) or with 2 X 10^{-8}M E_2 in the presence or absence of 5 μM LY, 50 μM PD098059 (PD), 2 μM ICI 182,780 (ICI), or 25 μM U0126 (U). The detection of total levels of PKB on the same blot controlled for loading discrepancies. B) Phosphorylated and total levels of PKB were assessed using the technique described above for cells treated with 2 X 10^{-8}M E_2 in the presence of 1, 5, 10, 25, or 50 μM LY294002.

Effect of E_2 on the Activation of the MAPK Cascade

As detailed in Section I, inhibition of the PI3-K and MAPK cascades prevented efficient E_2-induced cell cycle entry in MCF-7 cells. Several recent reports have demonstrated that the MAPK pathway is stimulated by E_2 in an ER-dependent fashion (Castoria et al., 1999; Endoh et al., 1997; Improta-Brears et al., 1999; Migliaccio et al., 1996; Watters et al., 1997). The dual specificity kinases, MEK1 and MEK2, phosphorylate a threonine and a tyrosine residue in the regulatory sites of Erk1 and Erk2 resulting in the activation of these MAPKs (reviewed in (Ahn et al., 1992). We performed a series of experiments to determine whether Erk1 and/or Erk2 were activated by E_2 under our conditions (Figure 4-2). The dually phosphorylated forms of Erk1 and Erk2 were specifically recognized on the immunoblot using a phospho-specific monoclonal antibody while total Erk1 and Erk2 was detected using a separate antibody after stripping and reprobing the same blot. In this case, activation was measured 5 minutes after treatment with either EGF, a known inducer of MAPK activity, or E_2. Compared to the untreated control (starved cells), 10 ng/ml EGF induced a dramatic increase in phosphorylated Erk1 and Erk2. Under the same conditions, 2 x 10^{-8}M E_2 had little or no effect on the levels of activated Erk1 or Erk2 (Figure 9A). A number of repetitions of this experiment failed to show more than a 1.5-fold induction. Neither ICI 182,780 nor LY294002 had any effect on active MAPK levels in E_2-stimulated cells. However, while E_2 failed to activate MAPK, the MAPK inhibitors (PD098059 and U0126) effectively reduced the basal levels of phospho-Erk1 and -Erk2.

The ability of U0126 and PD098059 to inhibit basal levels of activated MAPKs as well as cell cycle progression prompted me to examine if a correlation exists between these two activities. Using the same antibodies described above, MCF-7 cells were stimulated with E_2 for 5 minutes in the presence of variable concentrations of PD098059 or U0126 (Figure 9B). E_2 had no effect on Erk1 or Erk2 levels, but increasing concentrations of these compounds inhibited phosphorylation of these kinases. A clear reduction in phospho-Erk1 and -Erk2 was noted with as little as 1 μM U0126 compared to starved or E_2-treated cells. Peak inhibition was achieved between 5-10 μM. PD098059 also reduced the levels of active Erk1 and Erk2 with maximum inhibition reached at approximately 10 μM. Even at the highest concentration tested (50 μM), PD098059 was unable to completely inhibit phospho-Erk1/2 levels. These

data are consistent with relative S-phase inhibition (Figure 1B) observed with these two compounds and suggest that maintenance of basal levels of phospho-Erk1 and –Erk2 may be important in E_2-mediated mitogenesis.

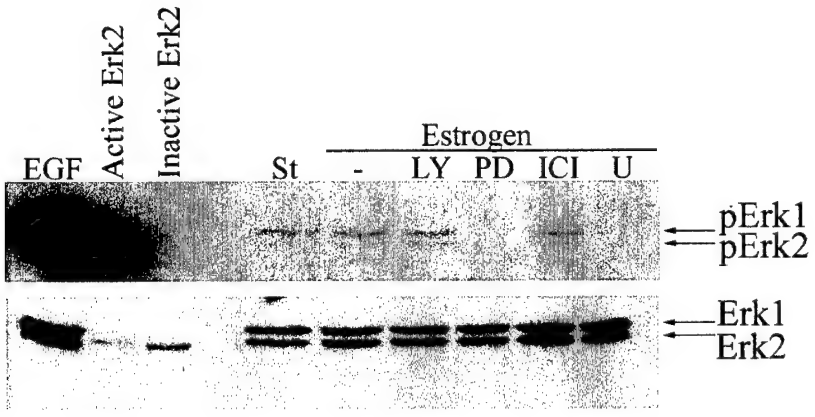
Effect of Culturing Conditions on E_2's Ability to Activate MAPK

Several publications describe contradictory findings regarding the ability of E_2 to stimulate activation of Erk1 and Erk2 in breast epithelial cells (Bonapace et al., 1996; Improta-Brears et al., 1999; Migliaccio et al., 1996). Variations in the methodology detailed in these studies include E_2 and serum concentrations, cell density, and the method of hormone delivery (Bonapace et al., 1996; Endoh et al., 1997; Improta-Brears et al., 1999; Migliaccio et al., 1996; Ruzycky, 1996; Watters et al., 1997). In order to determine which, if any, of these parameters could account for the varied results, I explored the effect of each condition on the ability of E_2 to stimulate MAPK activation.

First, I assayed the effect of variable E_2 concentrations on the phosphorylation of Erk1 and Erk2 in MCF-7 cells (Figure 10A). Cells were starved for 24 hours and then treated with varying amounts of E_2 by diluting the hormone in 0.1 mls of fresh starvation medium and then adding this to the existing culture media. Using immunoblotting, it was readily apparent that EGF induced a significant increase in the levels of phospho-Erk1 and –Erk2 levels as compared to the basal levels found in serum-starved cells. However, concentrations of E_2 ranging from 2×10^{-7} to 2×10^{-9}M did not elevate phosphorylated Erk1 and Erk2 above basal levels.

Several studies have detailed varying durations of starvation prior to hormone stimulation (Improta-Brears et al., 1999; Migliaccio et al., 1996). Increasing the period of starvation to 72 hours did not affect the levels of phospho-Erk1 or –Erk2 present after 5 minutes of E_2 treatment (data not shown). These results are consistent with our previous observations, but contradictory published reports led me to search further for MAPK activation.

A.



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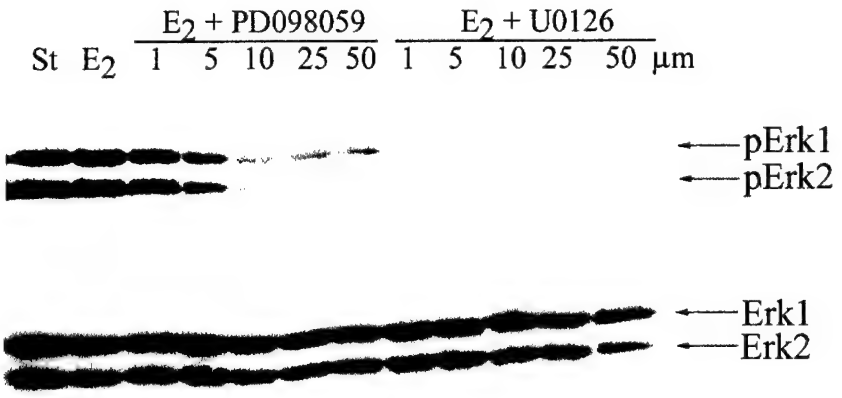
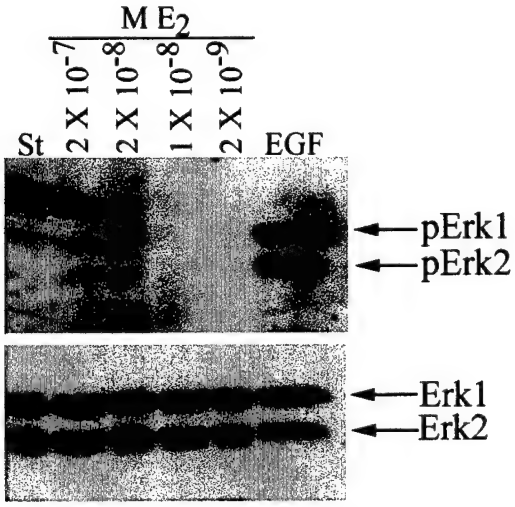


Figure 9. Effect of E_2-stimulation on MAPK phosphorylation status. A) The phosphorylation status of Erk1 and Erk2 was assessed using a phospho-specific antibody and immunoblot analysis on lysates from cells treated with 10 ng/ml EGF or 2 X 10^{-8}M E_2 in the presence or absence of 5 μM LY294002 (LY), 50 μM PD098059 (PD), 2 μM ICI 182,780 (ICI), or 25 μM U0126 (U). Levels of total Erk1 and Erk2 were detected on the same blot to control for loading variations. B) Phosphorylated and total levels of Erk1 and Erk2 were assessed using the technique described above for cells treated with 2 X 10^{-8}M E_2 in the presence of 1, 5, 10, 25, or 50 μM PD098059 or U0126.

A.



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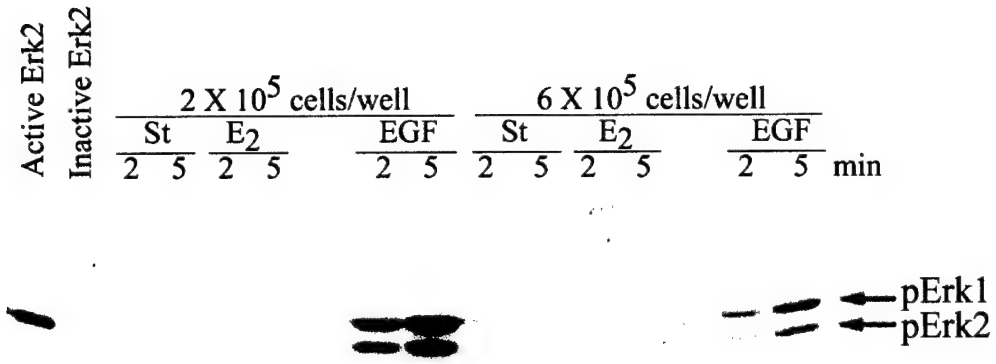


Figure 10. Effect of E_2 stimulation on the activation state of MAPK in MCF-7 cells. A) MCF-7 cells were stimulated (using the "add-to" method) with 10 ng/ml EGF or variable concentrations of E_2 (ranging from 2 X 10^{-9}M to 2 X 10^{-7}M) for 5 minutes. Cell lysates were fractionated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with a monoclonal antibody against diphosphorylated Erk1 and Erk2 (pErk1 and pErk2). The blot was stripped and total levels of Erk1 and Erk2 were detected using an antibody recognizing Erk2 that has cross-reactivity with Erk1. The serum-starved (St) negative control was mock treated with 100 µl of starvation medium. B) MCF-7 cells were seeded either at a low density (2 X 10^5 cells/well in a six-well dish) or a high density (6 X 10^5 cells/well). Cells were treated (using the "add-to" method) with EGF or E_2. Cells were lysed after 2 or 5 minutes of stimulation and total levels of Erk expression and the amount of phosphorylation was determined as described above. Commercially available positive and negative controls for MAPK phosphorylation, active Erk2 and inactive Erk2 (New England Biolabs), were included on this Western.

The activity of p38 kinase (a MAPK family member) may be modulated by cell density (Hines et al., 1999). Therefore, I explored the effect of confluency on the ability of E_2 to activate Erk1 and Erk2 (Fig. 4-10B). MCF-7 cells were seeded at a density of either 2 X 10^5 (subconfluent) or 6 X 10^5 (confluent) cells per well in 6-well dishes. Each well was treated with 2 X 10^{-8} M E_2 or 10 ng/ml EGF for 2 or 5 minutes. EGF stimulated the phosphorylation of Erk1 and Erk2 regardless of cell density though the magnitude of induction was significantly greater in the subconfluent cultures. E_2 did not measurably stimulate MAPK activity at either density; however, the basal levels of phospho-Erk1 and -Erk2 were slightly higher in confluent cultures.

In all of the experiments presented thus far, cultures have been stimulated in serum-free medium with no added growth factors. In other similar studies, hormone-depleted (charcoal-stripped) fetal bovine serum (CS-FBS) was present in culturing medium during E_2 stimulation, raising the possibility that co-factors found in serum may be necessary for the MAPK response (Improta-Brears et al., 1999; Migliaccio et al., 1996). To test this possibility, MCF-7 cells were starved and treated in medium containing previously reported concentrations of CS-FBS (0, 0.05, or 5%) (data not shown). Under these conditions, EGF increased phospho-Erk1 and –Erk2 to identical levels regardless of serum concentrations; however, E_2 failed to stimulate MAPK activation in all serum concentrations tested.

In searching for differences that might account for the discrepant observations, I also investigated the method of hormone delivery. In my previous experiments, the hormone was added without changing the medium ("add-to"). I compared this mode of delivery to replacing the medium at the time of activation with pre-warmed medium containing the hormone ("change"). Using the "change" technique, I observed increasing levels of active MAPK (Figure 4-11). However, when compared with the appropriate starvation control (St, No Drug, Change) it is apparent that the increase is not a result of E_2 stimulation, but rather an effect generated by replacing the medium. The increase in basal levels of phosphorylated MAPK was consistently observed, though the absolute level of increase was variable between experiments. Induction of MAPK due to media change, either with or without E_2, was not inhibited by the pure anti-estrogen, ICI 182,780, further indicating that this response is independent of ER signaling. Without appropriate controls, adding E_2 by changing the culture medium may be mistaken for specific activation of MAPK.

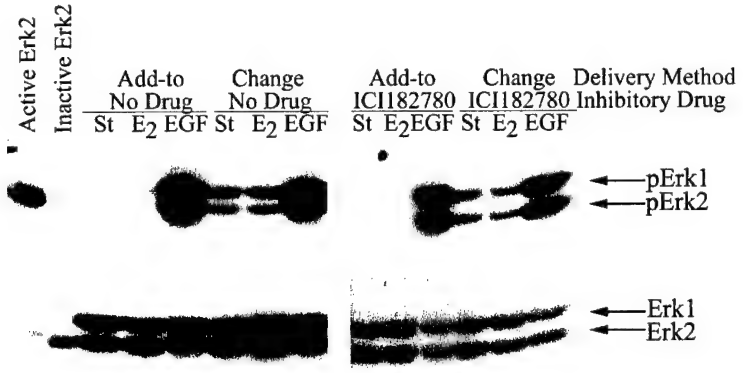


Figure 4-11. Effect of an ER antagonist on EGF- or E_2-induced phosphorylation of MAPK. Serum-starved MCF-7 cells were pretreated with 2 μM ICI 182,780 (ER antagonist) for 2 hours prior to hormone or growth factor stimulation. For "add-to" lanes, 100 μl of fresh starvation medium containing sufficient EGF or E_2 to generate a final concentration of 10 ng/ml or 2 X 10^{-8}M, respectively, was pipetted into each well. For "change" samples, starvation medium was aspirated and replaced with fresh medium containing the appropriate concentrations of both mitogen and inhibitor. After 5 minutes of stimulation, cells were lysed. Total and phosphorylated levels of Erk1 and Erk2 were visualized using Western analysis.

The Role of Autocrine Loops in E_2-Induced Proliferation

In Section I, I demonstrated that the addition of pharmacological inhibitors of MAPK activation (PD098059 and U0126) up to 6 hours after E_2 stimulation prevents mitogenesis (Figure 6). Considering the absence of measurable MAPK activation by E_2, I theorized that MAPK activity might be necessary as a delayed early event induced via an autocrine loop similar to that observed in human umbilical vein endothelial cells (HUVEC) (Kim-Schulze et al., 1998). To test this hypothesis, I used medium taken from hormone stimulated cells to re-stimulate fresh cultures (Figure 4-12A). Consistent with the transient nature of the mitogenic MAPK response, after 3 hours of stimulation, we observed a very minor increase in phospho-Erk1 and -Erk2 levels only in the EGF-stimulated cultures. When EGF-conditioned medium was transferred to untreated cells, the level of active MAPK dramatically increased, likely due to the continued presence of EGF in the medium. The transfer of conditioned medium from serum-starved cells stimulated a minor increase in the basal levels of active Erk1 and Erk2. This activation is likely an artifact resulting from the medium change (as shown in Figure 4-11) rather than the presence of a specific

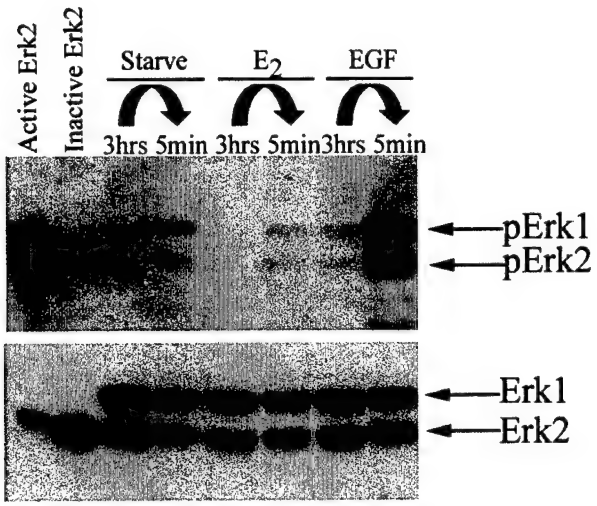
activator of MAPK in the starvation medium. Conditioned medium from E_2-treated cells had no effect on the levels of phospho-Erk1 and Erk2 when compared to the levels present in cells treated with conditioned starvation medium for 5 minutes. Conditioned medium from the E_2-stimulated cells was still capable of stimulating ER-mediated transcription and inducing proliferation (data not shown), indicating that the hormone was still bioactive, just without a detectable ability to activate MAPK.

We further explored the time course for activation by inducing MCF-7 cells with E_2 and harvesting cell lysates every 15 minutes for 4 hours (Figure 4-12B). At no time were the levels of phospho-Erk1 and –Erk2 elevated beyond basal levels. These data indicate no significant MAPK activation resulting from autocrine loops or directly from long-term E_2 treatment.

In order to further characterize the potential role of growth factors produced in response to E_2 stimulation, I examined whether their secretion was essential for E_2-induced mitogenesis. Previous work demonstrated that an IGF-I antibody could prevent IGF-I-induced mitogenesis, but did not inhibit the proliferative effect of E_2 on MCF-7 cells (Arteaga and Osborne, 1989). However, IGF-I is not the only growth factor expressed as a result of E_2 exposure in MCF-7 cells. Others include EGF and bFGF (Dickson et al., 1986; El Yazidi et al., 1998). The ability of neutralizing antibodies directed against these factors to inhibit E_2-mediated growth was tested (Figure 4-13). MCF-7 cells treated with EGF increased the population of cells in S phase greater than 4-fold. When co-incubated with either a neutralizing EGF antibody or an antibody recognizing the EGF receptor (EGFR), proliferation rates were maintained at basal or near-basal (starved) levels. Consistent with published data, an IGF-I neutralizing antibody prevented IGF-I induced mitogenesis, as did a bFGF antibody on bFGF-stimulated growth (Arteaga and Osborne, 1989). However, none of these antibodies affected E_2's ability to stimulate mitogenesis. While these studies do not exclude an autocrine growth factor loop as the basis for E_2-induced proliferation, the weight of evidence indicates that it is unlikely to be a major component of this process.

There are additional sites for potential interaction between E_2 and the cell surface. A small subpopulation of ER may localize to the plasma membrane (Razandi et al., 1999), and E_2 has been reported to bind to erbB2, a member of the class I family of receptor tyrosine kinases (Matsuda et al., 1993). However, neither an erbB2 receptor antibody (Herceptin) nor an antibody recognizing ER affected E_2-mediated proliferation (Figure 4-13).

A.



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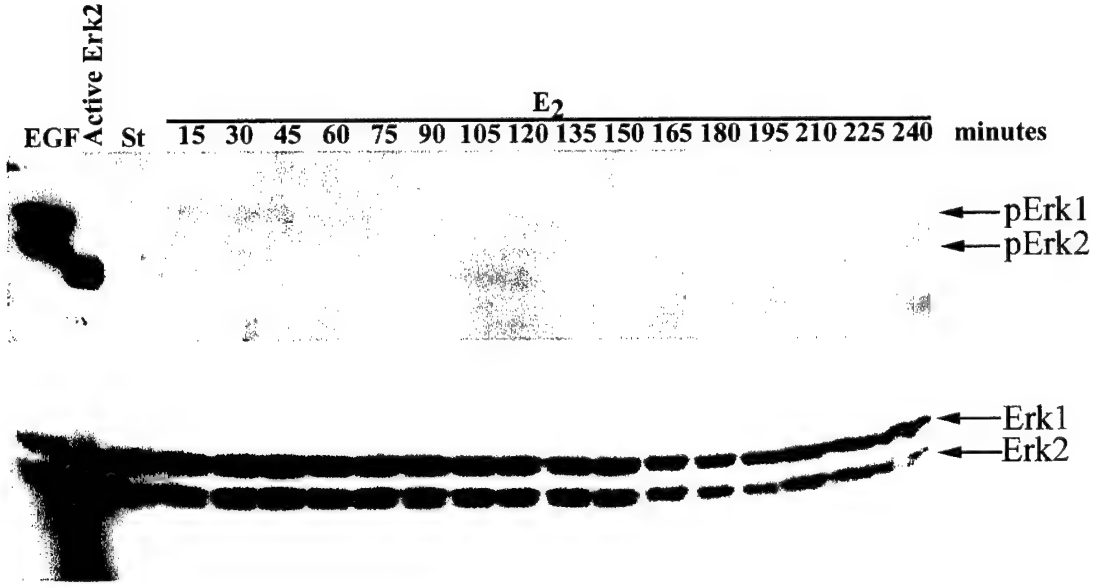


Figure 4-12. Effect of conditioned medium from MCF-7 cells treated for 3 hours with E_2 on the phosphorylation status of Erk1 and Erk2. A) Serum-starved MCF-7 cells were stimulated with 10 ng/ml EGF or 2 X 10^{-8}M E_2 at 37°C in 95% O_2/5% CO_2. After 3 hours this conditioned medium was transferred to a fresh well of cells and incubated at room temperature. Five minutes later cells were lysed and the total and phosphorylated levels of Erk1 and Erk2 were assessed using Western analysis. The minor increase in active Erk1 and Erk2 levels present in cells treated for 5 minutes with conditioned medium from starved or E_2-treated cells is likely to be an artifact of the media change (as seen in Figure 4-4) rather than the presence of a specific activator in the medium. B) Serum-starved MCF-7 cells were stimulated with 2 X 10^{-8}M E_2 for 4 hours. Time points were taken every 15 minutes and the total and phosphorylated levels of Erk1 and Erk2 were examined as detailed above.

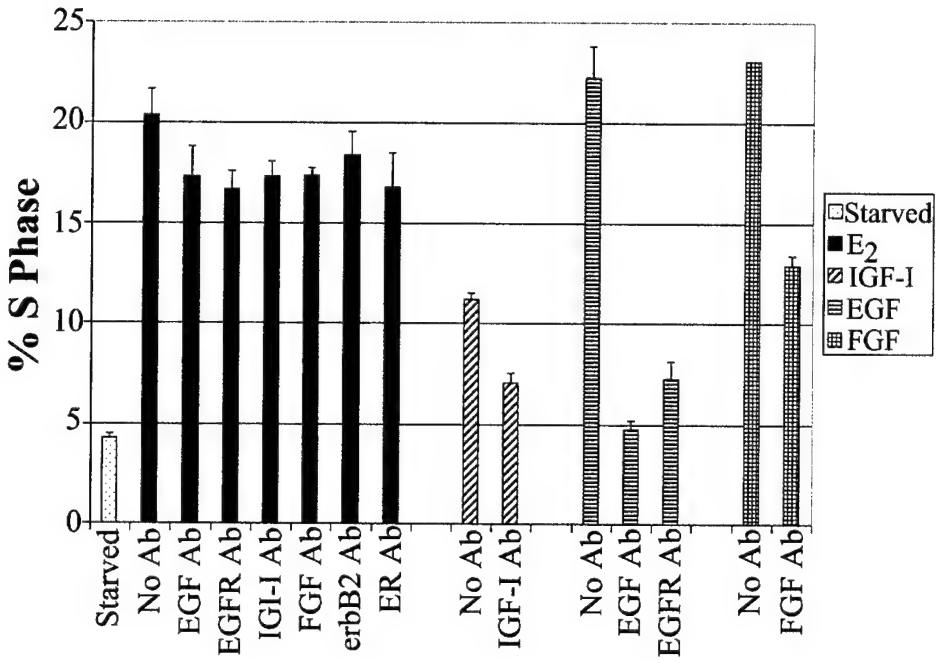


Figure 4-13. Effect of anti-growth factor antisera on E_2-induced cell cycle progression. Serum-starved MCF-7 cells were stimulated with 2 X 10^{-8}M E_2 or other growth factors in the presence or absence of 10 μg/ml EGF, EGFR, IGF-I, FGF, erbB2 or ER antisera for 24 hours. Cells were harvested, fixed, and stained. The S phase population was determined using flow cytometry. The data represent an average of results obtained from three experiments. *Bars*, SD.

Concentration of E_2 Necessary for Transcription and Proliferation

It has been reported that NIH-3T3 cells expressing transcriptionally inactive ER still proliferate in response to E_2, thereby uncoupling ER-mediated transcription from E_2-induced proliferation (Castoria et al., 1999). Therefore, one might expect a different dose-response for these two activities. Consistent with published reports, concentrations greater than 2 X 10^{-11}M increased the number of cells in S phase approximately 2.5-fold (Figure 4-7) (Yue et al., 1998). A minor increase in S phase population was observed in cells treated with 2 X 10^{-12}M E_2. This demonstrates that MCF-7 cells respond mitogenically to physiologic doses of E_2 though activation of MAPK is not detected (Figures 10A and 4-14).

To assess the concentration of E_2 necessary for a transcriptional response, I measured the dose-response using three independent measures of ER-mediated transcription: c-myc expression, ERE-luciferase reporter gene activity, and pS2 levels. c-myc is an immediate early gene that contains an ERE in its promoter (Dubik and Shiu, 1992). Using Northern analysis, c-myc transcript levels were measured in MCF-7 cells after 1 hour of hormone stimulation (Figure 4-15). Treatment with concentrations between 2 X 10^{-12}M and 2 X 10^{-8}M E_2 resulted in increased c-myc expression as compared with the basal levels found in serum-starved cells. Less concentrated doses had no transcriptional effects, consistent with mitogenic concentrations of the hormone.

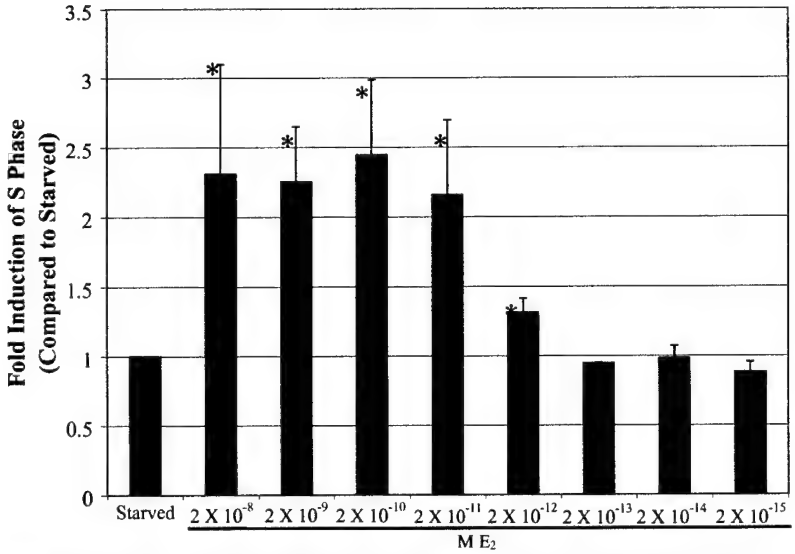


Figure 4-14. Dose response of E_2 on induction of mitogenesis. Serum-starved MCF-7 cells were stimulated with variable concentrations of E_2 (2×10^{-8}M to 2×10^{-15}M) for 24 hours. Cells were harvested, fixed, and stained. The S-phase population was determined using flow cytometry. The data represent an average of results obtained from three experiments. * denotes statistically significant increase in the percentage of cells in S phase at $p \leq 0.05$. *Bars*, SD.

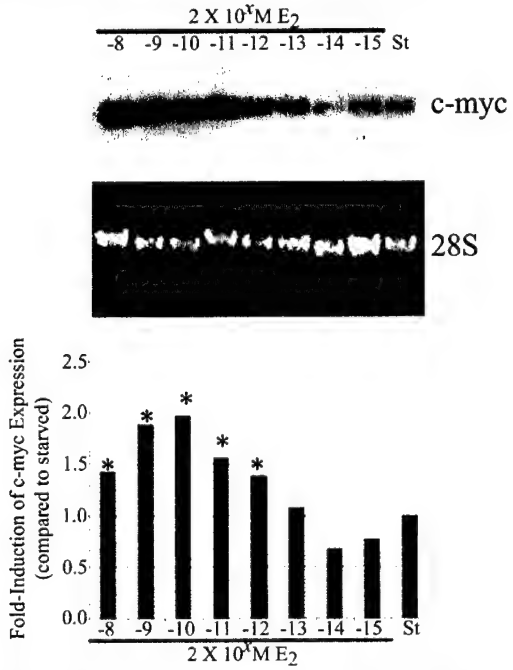


Figure 4-15. Dose response of E_2-induced transcription of the immediate early gene c-myc. Representative Northern blot analysis of c-myc RNA expression in MCF-7 cells treated for 1 hour with different concentrations of E_2 (2×10^{-8}M to 2×10^{-15}M). The starved lane displays the amount of c-myc RNA present at time 0. Levels of the 28S ribosomal subunit were used as an internal control to correct for loading inconsistencies. * denotes statistically significant increase in myc transcript levels at $p \leq 0.05$.

Since c-myc is an immediate early gene, its expression is upregulated in response to a variety of mitogenic signals and pathways. Therefore, expression of this gene may be indirectly responsive to E_2. For this reason, I measured the transcriptional response to E_2 stimulation using a transient reporter system (Figure 4-16). MCF-7 cells were transfected with one of two luciferase constructs containing E_2-responsive promoters (TK-ERE, vitellogenin promoter, or 3X-ERE, three consensus EREs). Transfected cells were treated with serial dilutions of E_2 (2×10^{-8}M – 2×10^{-15}M) for 24 hours prior to lysis and assay of luciferase activity. E_2 concentrations greater than 2×10^{-10}M stimulated an approximately 4-fold increase in luciferase activity compared with serum-starved cells. 2×10^{-11}M E_2 almost doubled the amount of luciferase activity, whereas lower concentrations had no effect. Again, the concentration of E_2 sufficient to elicit a detectable transcriptional response correlates with the dose necessary for proliferation.

The pS2 gene was identified as being E_2-responsive in a screen of rapidly induced transcripts in E_2-treated MCF-7 cells. pS2 does not require *de novo* protein synthesis for its expression, and contains a functional ERE in its promoter (Brown et al., 1984; Cavailles et al., 1989; Masiakowski et al., 1982; Nunez et al., 1989). Also, in clinical breast cancer samples, pS2 expression is correlated with responsiveness to hormone therapy and favorable tumor characteristics (Balleine and Clarke, 1999). Transcript levels of the pS2 gene were measured after 24 hours of stimulation with variable concentrations of E_2 (Figure 4-17). Expression of pS2 more than doubled in response to concentrations of E_2 greater than 2×10^{-11}M. Consistent with proliferation and c-myc expression, 2×10^{-12}M produced a small but significant effect on pS2 transcription. These three independent assays of transcriptional activity define the minimal concentration of E_2 sufficient to modulate gene expression. This dose is equivalent to the concentration necessary for inducing cell cycle progression in wild-type MCF-7 cells, indicating that these two processes are tightly linked.

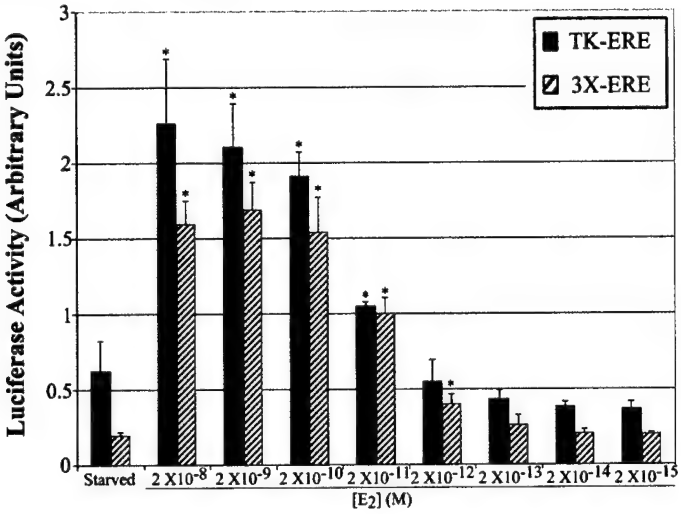


Figure 4-16. Dose response of E_2-induced ER-mediated transcription. MCF-7 cells were transfected with a luciferase construct containing either an artificial (3X-ERE) or a naturally occurring (TK-ERE) E_2-responsive promoter. After 24 hours of stimulation with varying concentrations of E_2 (2×10^{-8}M to 2×10^{-15}M), samples were harvested, and the luciferase activity was measured. Corrections for differences in protein concentration and transfection efficiencies were made using detected levels of a CMV-luciferase construct from a different species. The average of the normalized activity for three experiments is given. * denotes statistically significant increase in luciferase activity at $p \leq 0.05$. *Bars,* SD.

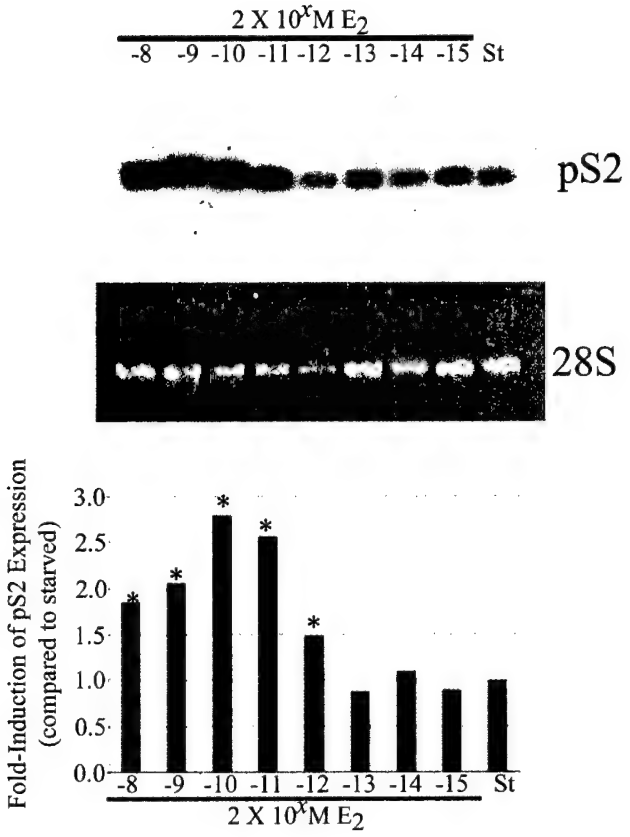


Figure 4-17. Dose response of E_2-induced transcription of pS2. Representative Northern blot of pS2 RNA expression in MCF-7 cells treated for 24 hours with different doses of E_2 (2×10^{-8}M to 2×10^{-15}M). Basal levels of pS2 RNA are represented in the starved lane (time 0). Loading discrepancies were corrected by measuring differences in the level of the 28S ribosomal RNA present in each lane. * denotes statistically significant increase in pS2 transcript levels at $p \leq 0.05$.

Discussion

ER-mediated transcription is generally accepted as the molecular mechanism that enables a proliferative response to E_2 stimulation (Musgrove and Sutherland, 1994). However, the recent report by Castoria *et al.* raises significant questions about this model, (Castoria et al., 1999). That study used NIH-3T3 cells transiently transfected with a transcriptionally inactive ER construct (lacking the DNA binding domain). Surprisingly these cells proliferated when treated with E_2, suggesting either an error in the existing model or the presence of an alternative pathway capable of mediating cell cycle progression. Activation of MAPK has been proposed as this alternate pathway.

I investigated the role of MAPK and PI3-K in E_2-induced mitogenesis and demonstrated that E_2 treatment of MCF-7 cells stimulates cell cycle progression in the absence of detectable MAPK or PKB activation. Further studies revealed that activation of MAPK in response to E_2 treatment does not occur, regardless of E_2 or serum concentrations, cell density, or the method of hormone delivery. These studies added support to the theory that growth factor production resulting from E_2 stimulation does not stimulate

an autocrine loop essential for the mitogenic response in MCF-7 cells (reviewed in (van der Burg et al., 1992). Since ER-mediated transcription may not be necessary for E_2-induced mitogenesis, as proposed by Castoria *et al.,* I attempted to uncouple the transcriptional activity of E_2 from its proliferative action based on concentration differences. I found that a minimal E_2 concentration of 2×10^{-12}M is needed to induce measurable transcription as well as stimulate cell cycle progression. Cumulatively, these findings support the theory that ER-mediated transcription directly regulates E_2-stimulated mitogenesis.

Some previous studies have supported the observation that E_2 stimulation does not activate MAPK in MCF-7 cells (Bonapace et al., 1996). However, other studies have described activation (Castoria et al., 1999; Improta-Brears et al., 1999; Migliaccio et al., 1996). The reported discrepancy may be the result of genetic differences between MCF-7 cells maintained in different laboratories. Therefore, a culture of MCF-7 cells maintained in a separate laboratory was also tested and no MAPK activation was detected in these cells either (data not shown). Under all conditions examined we did not detect activation of MAPK in response to E_2 treatment though mitogenesis still resulted.

This study raises questions regarding the role of MAPK in E_2-induced mitogenesis. The lack of any reproducible induction of MAPK activity as measured by Erk1 and Erk2 phosphorylation contrasts with several published reports (Castoria et al., 1999; Endoh et al., 1997; Improta-Brears et al., 1999; Migliaccio et al., 1996; Watters et al., 1997). Published accounts of this activation have also shown a relatively modest induction (between 1.5 and 3-fold) over control values (Endoh et al., 1997; Migliaccio et al., 1996). In all reported cases and in my experiments, the magnitude of activated MAPK resulting from a cell surface signal such as EGF far exceeds any induction due to E_2 (Improta-Brears et al., 1999). Furthermore, E_2 can overcome a cell cycle block induced by Simvastatin, an HMG-CoA reductase inhibitor, without detectable MAPK activation (Bonapace et al., 1996). It is also clear from my experiments that E_2 is mitogenic in the absence of measurable MAPK activation, which raises the issue of the physiologic relevance of the observed response.

Though detectable activation of MAPK does not occur in response to E_2 stimulation, I had previously demonstrated that inhibition of the MAPK cascade arrests E_2-treated MCF-7 cells in the G1 stage of the cell cycle (Figure 1A). This suggested two alternate hypotheses: (a) MAPK was activated in a delayed fashion, perhaps through the stimulation of growth factor synthesis inducing an autocrine loop, or (b) basal activity of MAPK is necessary for cell cycle progression in MCF-7 cells. The results in this chapter suggest that the first hypothesis is unlikely. MAPK activity was not stimulated by conditioned medium from E_2-treated cells. Further, neutralizing antibodies to IGF-I, EGF, EGFR, FGF, and ErbB2 failed to inhibit E_2 mediated growth. These findings suggest that basal levels of active MAPK are essential to maintain cells in a "receptive state" for mitogenic stimulation and that depletion of these signaling intermediates functions as a dominant negative influence on cell cycle progression.

These studies demonstrate that activation of MAPK does not occur in response to E_2 stimulation of MCF-7 cells. Furthermore, the production of IGF-I, bFGF, and EGF does not play an essential role in E_2-induced mitogenesis. Finally, the roles of E_2 as a mitogen and transcription factor could not be uncoupled based on concentration. Cumulatively these data indicate that MAPK activation is not an essential feature of E_2-stimulated proliferation and further support the central role of ER-mediated transcription in this process.

Section III

Introduction

MCF-7 cells are not the only immortalized cells that respond to E_2's transcriptional and proliferative effects, yet they were the only cells used in the previous experiments. Therefore, I could not

exclude the possibility that E_2 causes cell growth through MAPK and PI3-K signaling in MCF-7 cells alone. I thus explored whether E_2 stimulates cell growth via MAPK and PI3-K signaling in the following cell types: ZR75-1, another hormone responsive breast cancer cell line; Ishikawa, an endometrial adenocarcinoma line; 26NC, DU99, and BE-20E6, three immortalized normal breast epithelial cells lines; and 40N, 43N, 45N, and 48N, four primary normal breast epithelial lines.

There is evidence suggesting that ZR75-1 and MCF-7 cells share a similar response to E_2. Both cell lines overexpress ER compared to normal breast epithelial cells, but ER expression is higher in MCF-7 cells. E_2-treated ZR75-1 and MCF-7 cells progress through the cell cycle with similar kinetics (Osborne et al., 1984). In both ZR75-1 and MCF-7 lines, E_2 is also sufficient to overcome a cell cycle block generated by tamoxifen, a partial estrogen antagonist (Osborne et al., 1984). Finally, E_2 stimulation of two lines has demonstrated similar effects on gene expression (Mathieu et al., 1991; Rochefort et al., 1989). Cumulatively, these findings indicate that the ZR75-1 cells, like MCF-7 cells, are breast cancer cells that activate transcription and proliferate in response to E_2 treatment.

Ishikawa cells can proliferate in E_2-free medium, suggesting that these cells are not hormone dependent (Nishida et al., 1985). However, E_2 stimulation generates a detectable increase in the level of PR expression as well as alkaline phosphatase activity, both of which have been reported as E_2-responsive genes (Holinka and Gurpide, 1979; Holinka et al., 1986b; Holinka et al., 1986c; Romano et al., 1989). When Ishikawa cells are treated with E_2, their DNA polymerase α activity increases 4- to 5-fold and they progress through the cell cycle (Gravanis and Gurpide, 1986; Holinka et al., 1986a). Thus even though Ishikawa cells are not hormone dependent, they are another example of cancerous cells that activate transcription and proliferate in response to E_2 treatment.

Cancerous cells were not the only breast epithelial cells in which I investigated the roles of PI3-K and MAPK in cell growth. I also examined these signals in immortalized normal mammary epithelial, such as 26NC cells. The 26NC line was established by immortalizing a culture of normal breast epithelial cells (26N) with DMBA. Chemical immortalization of breast epithelial cells can result in increased expression of the ER-β isoform (Hu et al., 1998). Because increased receptor expression could cause artifactual findings, I used two additional lines that were immortalized without chemicals. The DU99 line was made by transfecting normal breast epithelial cells with an expression construct for the catalytic subunit of the human telomerase gene. The BE20-E6 breast cell line was immortalized by stably transfecting the human papilloma virus E6 gene into normal breast epithelial cells.

In order to study the role of MAPK and PI3-K in the proliferation of breast epithelial cells in the continuum of stages involved in transformation, I also examined cultures of primary normal epithelial cells. Dr. Gregory S. Georgiade had obtained breast tissue from reduction mammoplasty from four separate patients: a 34 year old (40N), a 15 year old (43N), a 18 year old (45N), and a 19 year old (48N). The tissue was treated with collagenase and hyaluronidase to digest the extracellular matrix and the stromal components were removed by filtration (Stampfer et al., 1980). The remaining cells are a relatively pure population of normal breast epithelial cells.

In the following experiments, I examined the role of MAPK and PI3-K signaling after E_2 and IGF-I treatment of transformed, immortalized, and normal breast epithelial cells. Growth factor, but not E_2, cause immortalized and normal breast epithelial cells to proliferate, and this proliferation requires MAPK and PI3-K activity. Both E_2 and IGF-I cause hormone-responsive transformed breast cells to proliferate, and again this proliferation required the MAPK and PI3-K signaling cascades. Yet neither MAPK nor PI3-K activation was detected in response to E_2. This suggests that basal levels of MAPK and PI3-K activity are necessary for E_2-induced cell cycle progression.

Effect of Pharmacological Inhibitors on the Viability of ZR75-1 and Ishikawa Cells

The data presented in Sections I and II indicate a requirement of basal MAPK and PI3-K activity for a mitogenic response to E_2. To corroborate these findings, I sought to reproduce the results in two other E_2-responsive cell lines, the breast cancer epithelial cell line ZR75-1 and the Ishikawa endometrial adenocarcinoma line. Inhibitor concentrations that did not demonstrate cytotoxic effects on either cell line were identified by a flow cytometric viability assay (Table 2). Cells were treated for 48 hours with variable drug concentrations. The viability profiles for each compound in both cell lines were remarkably similar to each other and the MCF-7 cells. Therefore, the identical inhibitor concentrations employed in the MCF-7 studies were chosen for further experimentation on ZR75-1 and Ishikawa cells.

Percentage of Viable Cells

Condition	*Dose*	ZR75-1	Ishikawa
Log		98.4	98.1
Starved	Starved	96.6	94.0
U0126	5 μM	85.5	78.6
	10 μM	90.0	74.1
	25 μM	88.1	86.1
	50 μM	81.9	43.7
	100 μM	9.7	19.8
PD098059	5 μM	94.5	93.1
	10 μM	95.0	93.8
	25 μM	94.5	92.9
	50 μM	94.9	89.3
LY294002	5 μM	93.9	91.9
	10 μM	89.2	91.4
	25 μM	85.2	78.1
	50 μM	81.9	65.2
	100 μM	78.4	46.5
H-89	500 nM	94.7	94.4
	1 μM	95.4	92.8
	10 μM	89.1	88.3
ICI182,780	2 μM	94.7	89.7
	20μM	94.2	89.6

Table 2. ZR75-1 and Ishikawa cell viability in the presence of variable concentrations of pharmacological inhibitors. ZR75-1 and Ishikawa cells were treated with the different dosages of U0126 (U), PD098059 (PD), LY294002 (LY), H-89, or ICI 182,780 (ICI). After 48 hours cells (both adherent and floating) were harvested and resuspended in complete medium containing propidium iodide. The cells were sorted by flow cytometry to determine the percentage of cells that excluded the dye (viable).

Effect of MAPK and PI3-K Inhibitors on E_2-Induced Mitogenesis in ZR75-1 Cells

Before continuing with the inhibitor studies as described earlier on the MCF-7 cells, I first determined the ideal starvation conditions necessary to arrest ZR75-1 and Ishikawa cells in the G1 phase of the cell cycle. Cells were seeded and allowed to adhere to the dish for 24 hours prior to the removal of

the conditioned medium and addition of starvation medium. Cells were then starved for 24, 48, 72 or 96 hours prior to either stimulation with 2 X 10^{-8}M E_2 or continued starvation. Under these parameters, the ZR75-1 line displayed a 2-fold increase in the percentage of cells undergoing DNA synthesis after 24 hours of serum-deprivation (Figure 18A). However, Ishikawa cells did not growth arrest even after 96 hours of serum starvation (Figure 18B). It had previously been demonstrated that E_2 stimulation of these cells did result in increased cell proliferation, but only after 10 days (Holinka et al., 1986a). Because the time interval required to detect measurable effects on kinase activation or expression of cell cycle components is critical for these experiments, I did not want to deviate from the experimental protocol developed for MCF-7 cells. For this reason, no additional experiments were performed using the Ishikawa line, but rather efforts focused on the effects of E_2 stimulation on ZR75-1 cells.

Next the rate of DNA synthesis was assessed in serum-starved ZR75-1 cells treated with E_2 in the presence or absence of inhibitors of MAPK, PI3-K, PKA, PKC or a pure anti-estrogen. Cell cycle distribution was measured after 24 hours of stimulation (Figure 18A). As mentioned earlier, E_2 stimulation resulted in a 2-fold increase in the population of cells in S-phase. The anti-estrogen (ICI) maintained the growth rate at starved levels, while neither the PKA (H-89) nor the PKC (BisI) inhibitors displayed any effect on E_2-induced cell cycle progression. Both inhibitory compounds of the MAPK cascade (PD and U) were as effective at blocking E_2 action as the anti-estrogen (ICI). The PI3-K inhibitor (LY) was even more potent at inhibiting E_2 action, and the percentage of cells in S phase was reduced below basal levels. These data suggest that the activities of both MAPK and PI3-K, but not PKA or PKC, are critical for the proliferative response of ZR75-1 cells to E_2 stimulation. While the potencies of different compounds varied slightly between ZR75-1 and MCF-7 cells, the overall trend was the same: PI3-K and MAPK activity are required for E_2-induced proliferation.

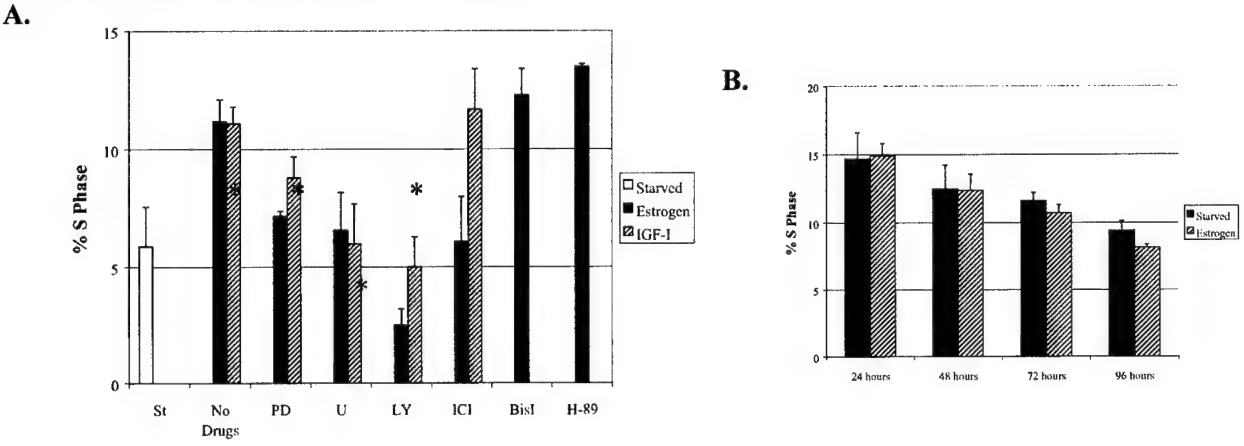


Figure 18. Effect of serum-starvation and E_2 stimulation on cell cycle progression in estrogen responsive cell lines. A) Percentage of ZR75-1 cells in S phase after 24 hours of serum-starvation prior followed by 24 hours of 2 X 10^{-8}M E_2 or 30 ng/ml IGF-I treatment in the absence (No Drugs) or presence of 50 μM PD098059 (PD), 10 μM U0126 (U), 5 μM LY294002 (LY), 2 μM ICI 182,780 (ICI), 500 nM bisindolylmaleimide I (BisI), or 500 nM H-89. * denotes statistically significant decrease in E_2-induced mitogenesis at $p \leq 0.05$. *Bars,* SD. B) Ishikawa cells were serum-starved for 24, 48, 72, or 96 hours. Cells were then stimulated with 2 X 10^{-8}M E_2 for 24 hours prior to measuring the rate of DNA synthesis. *Bars,* SD.

Inhibitor Effects on Cyclin D1 Expression, cdk2 Activation, and Rb Hyperphosphorylation in ZR75-1 Cells

As detailed in Section I, accumulation of cyclin D1, a key regulatory event in the G1 checkpoint, is potentially dependent upon the activity of MAPK and PI3-K. Using the same experimental procedures described for MCF-7 cells, I examined the expression of cyclin D1 as well as cdk2 activity and Rb phosphorylation status in ZR75-1 cells. Surprisingly, no increase in the levels of cyclin D1 protein occurred in response to E_2 treatment (Figure 5-19A). This finding contrasted results from MCF-7 cells as well as numerous publications detailing the accumulation of this protein in order for the G1/S transition to occur (Altucci et al., 1996; Foster and Wimalasena, 1996; Prall et al., 1997). Despite the lack of cyclin D1 accumulation, 24 hours of E_2 stimulation did result in an induction of cdk2 kinase activity and subsequent Rb phosphorylation (Figure 5-19B and data not shown). Consistent with their inhibitory effects on cell cycle progression, ICI 182,780, U0126, and LY294002 prevented the activation of cdk2 as well as Rb phosphorylation. Bisindolylmaleimide I and H-89 had no effect on the increase of cdk2 activity or phospho-Rb levels, consistent with cell cycle profiles in the presence of these compounds. Surprisingly, PD098059, which completely inhibited E_2-induced mitogenesis, displayed no inhibitory effects on cdk2 activation or Rb hyperphosphorylation. Aside from this intriguing finding with the PD098059 compound, the cell cycle effects of PKA, PKC, PI3-K and MAPK inhibitors on E_2-induced mitogenesis in ZR75-1 cells mirrored the findings obtained with MCF-7 cells. Collectively, these results support the theory that MAPK and PI3-K activities are essential in the proliferative response to E_2 either at, or prior to, the accumulation of cyclin D1.

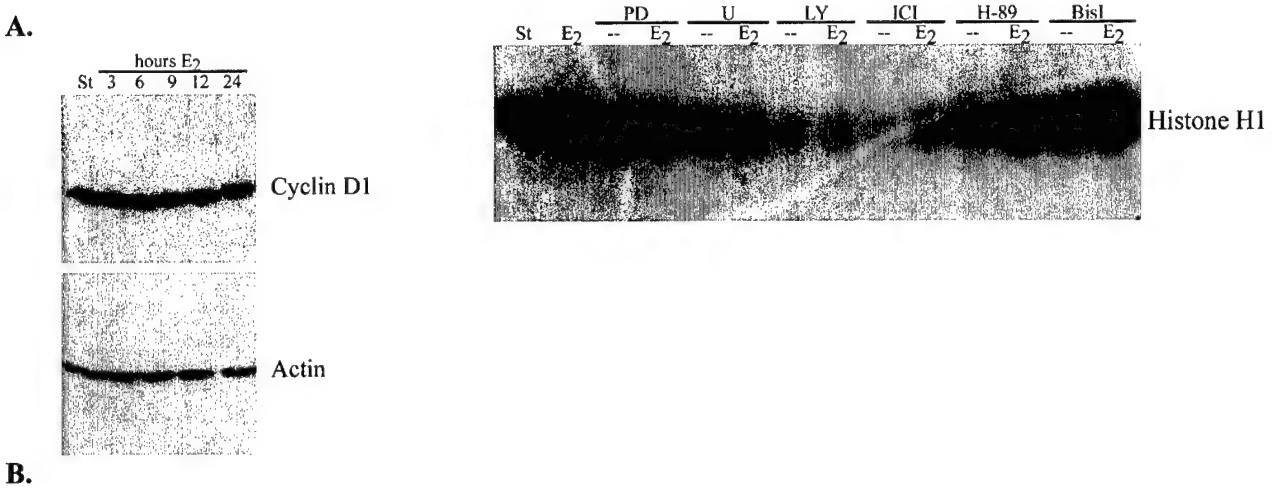


Figure 19. Effects of cytoplasmic signaling inhibitors on cyclin D1 expression and cdk2 activity in ZR75-1 cells. A) ZR75-1 cells were stimulated with E_2 for 3, 6, 9, 12, or 24 hours. Cells were lysed and relative amounts of cyclin D1 was detected by immunoblotting. Blots were reprobed for actin to control for loading variations. B) ZR75-1 cells were treated with 50 μM PD098059 (PD), 10 μM U0126 (U), 5 μM LY294002 (LY), 2 μM ICI 182,780 (ICI), 500 nM H-89, or 500nM bisindolylmaleimide I (BisI) in the absence (--) or presence of 2 X 10^{-8}M E_2. After 24 hours of treatment, cells were lysed and cdk2 was immunoprecipitated; the activation status of this kinase was assayed by determining its ability to phosphorylate histone H1.

Effect of E_2 on the Activation of the MAPK and PI3-K Cascades in ZR75-1 Cells

Inhibition of MAPK and PI3-K activity prevents E_2-induced cell cycle progression in both MCF-7 and ZR75-1 cells. Furthermore, E_2 fails to induce detectable activation of either cascade in MCF-7 cells. To determine if activation of PI3-K and PKB occurs in ZR75-1 cells, the assay was repeated using 2 X 10^{-8}M E_2 or 30 ng/ml IGF-I as a positive control in the presence or absence of pharmacological inhibitors of PKA, PKC, MAPK, or PI3-K. Dually phosphorylated forms of Erk1 and Erk2 were detected on an immunoblot using a phospho-specific antibody (Figure 20). E_2 failed to induce phosphorylation of Erk1 and Erk2, whereas IGF-I stimulated a significant increase. Activation of MAPK by IGF-I was prevented in the presence of either MAPK inhibitor (PD and U); however, incubation with a pure anti-estrogen (ICI) or a PI3-K inhibitor (LY) had no effect. Although E_2 did not induce activation of Erk1 or Erk2, the MAPK inhibitors did reduce the basal levels of active MAPK below the level of detection. This finding was consistent with results from MCF-7 cells, suggesting that maintenance of basal levels of phospho-Erk1/Erk2 may be a required for E_2-induced mitogenesis.

As detailed earlier, PKB, a downstream target of the PI3-K cascade, has been implicated in the stabilization of cyclin D1 protein, which is necessary for cell cycle progression (Muise-Helmericks et al., 1998). When ZR75-1 cells were treated with E_2 for 5 minutes, no detectable increase in the phosphorylation status of this kinase was detected (Figure 20). Conversely, IGF-I, a known activator of PI3-K in ZR75-1 cells (Jackson et al., 1998), generated a 5-fold increase in phospho-PKB levels, which was not inhibited by PD098059, U0126, or ICI 182,780. Incubation with 10μM LY294002 not only prevented the induction of PKB phosphorylation of IGF-I-treated samples, but also lowered the basal levels observed in E_2-stimulated cells. These results corroborate my findings in MCF-7 cells and indicate that basal levels of PI3-K activity may be essential for the mitogenic response to E_2.

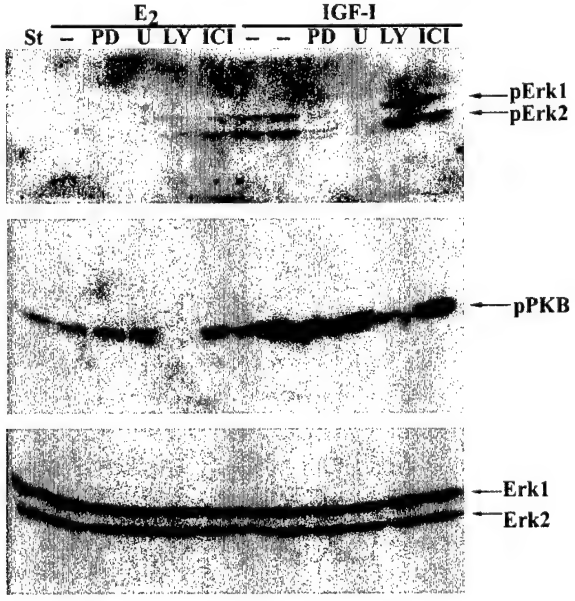


Figure 20. Effect of E_2 or IGF-I stimulation on MAPK and PKB phosphorylation status in ZR75-1 cells. A) The phosphorylation status of Erk1, Erk2, and PKB was assessed on lysates from ZR75-1 cells treated for 5 minutes with 30 ng/ml IGF-I or 2 X 10^{-8}M E_2 in the absence (--) or presence of 50 μM PD098059 (PD), 10 μM U0126 (U), 10 μM LY294002 (LY), or 2 μM ICI 182,780 (ICI). Phosphorylated levels of Erk1, Erk2, and PKB were assessed using phospho-specific antibodies. Total Erk1 and Erk2 expression levels were detected on the same blot to control for loading variations.

Viability of Immortalized and Normal Breast Epithelial Cells Treated with Pharmacological Inhibitors of Signaling Cascades

All of the experiments detailed thus far have explored the mitogenic response of E_2-responsive breast cancer cells. To extrapolate these findings, I next wanted to determine if the same requirements of cytoplasmic signaling cascade action exist for the proliferative response of normal breast epithelial cells. For these studies, I used three different immortalized normal lines (26NC, DU99, and BE20-E6) and four primary cultures (40N, 43N, 45N, and 48N) established from tissue obtained from reduction mammoplasties. Prior to exploring the capability of E_2 and IGF-I to activate MAPK in these lines, a flow cytometric viability assay was performed to examine the effects of the inhibitors on cell survival (Table 3). Cells were treated with variable concentrations of PD098059, U0126, LY294002, H-89, and ICI182, 780 for 48 hours. 26NC cells were slightly less viable in the presence of inhibitors compared to the other immortalized cell lines. 26NC cells may be more sensitive to chemical treatment due to the fact that this line was established via a chemical immortalization process, whereas the other lines were transfected with specific genes in order to generate immortalized cells. Overall, each compound generated viability profiles remarkably similar to those previously generated for the cancerous cell lines. The minor exception was 50 μM PD098059, which consistently displayed slightly elevated cytotoxic effects compared to the other compounds. Therefore, the identical inhibitor concentrations employed in the cancer studies were chosen for further experimentation with these normal lines, except in the case of 45N cells which required a lower dose of PD098059 (10 μM).

Percentage of Viable Cells

Condition	*Dose*	DU99	BE20	26NC	40N	43N	45N	48N
Log		99.3	97.5	83.8	97.6	93.8	96.4	98.5
Starved	Starved	96.7	88.7	80.3	97.5	87.0	83.0	91.9
U0126	5 μM	86.5	82.7	55.1	99.1	91.1	95.4	97.2
	10 μM	89.6	72.7	55.6	98.7	90.1	95.0	95.5
	25 μM	83.6	68.7	43.2	96.9	83.0	82.6	89.5
	50 μM	41.7	33.2	23.0	83.0	84.8	72.7	84.1
	100 μM	18.9	13.8	11.7	17.0	23.8	22.8	19.9
PD098059	5 μM	94.3	85.7	70.6	98.9	92.5	92.1	99.0
	10 μM	93.5	83.2	73.8	98.4	85.1	92.5	96.9
	25 μM	91.5	80.5	72.9	96.3	80.6	65.4	81.9
	50 μM	89.3	81.3	66.5	96.5	80.7	67.8	84.5
LY294002	5 μM	94.3	83.5	68.4	95.5	--	--	--
	10 μM	90.6	81.3	74.4	98.4	92.1	95.2	97.5
	25 μM	83.7	73.8	68.2	98.2	86.6	96.0	99.8
	50 μM	78.9	69.2	64.1	98.2	87.1	95.0	96.0
	100 μM	68.7	63.3	57.3	95.6	87.0	94.6	95.5
H-89	500 nM	97.1	88.2	82.5	97.6	94.1	93.3	99.0
	1 μM	94.9	86.5	85.3	98.8	94.5	96.9	99.0
	10 μM	86.4	45.4	80.0	96.2	75.9	62.6	91.7
ICI182,780	2 μM	93.3	87.3	76.2	92.2	84.1	71.7	84.3
	20μM	96.5	85.9	78.4	91.4	80.5	72.3	86.7

Table 3. Normal and immortalized cell viability in the presence of variable concentrations of pharmacological inhibitors. Cultures of primary normal breast epithelium (40N, 43N, 45N, and 48N) as well as immortalized normal cells (DU99, BE20-E6, and 26NC) were treated with the varying concentrations of U0126 (U), PD098059 (PD), LY294002 (LY), H-89, or ICI 182,780 (ICI) for 48 hours. Adherent and floating cells were harvested and stained with propidium iodide. The percentage of cells that excluded the dye (viable) was measured by flow cytometry.

Activation of MAPK in Response to Growth Factor or E_2 Treatment in Normal Breast Epithelial Cells

I next assessed the induction of MAPK activity in immortalized and normal mammary epithelial cells after stimulation with 2 X 10^{-8}M E_2 or 30 ng/ml IGF-I. After 5 minutes of stimulation, the presence of dually phosphorylated forms of Erk1 and Erk2 as well as total MAPK expression were assessed by immunoblotting. Consistent with the cancerous lines, none of the immortalized normal lines (26NC, DU99, and BE20-E6) demonstrated a significant response to E_2 stimulation (Figure 21). On the other hand, IGF-I, which activated MAPK in both MCF-7 and ZR75-1 cells, displayed variable results. No IGF-I-induced activation of MAPK was detected in the 26NC cells. Interestingly, Erk2 was dramatically induced compared to Erk1 levels in the BE20-E6 and DU99 cells, though complete inhibition occurred in the presence of PD098059 or U0126. Surprisingly, enhanced activation of MAPK in all three immortalized normals was displayed in samples derived from cells treated with bisindolylmaleimideI or H-89, indicating that either these drugs increased the basal levels of phosphorylated Erk1 and Erk2 or they are acting as co-activators of the response. However, in MCF-7 cells H-89 and bisindolylmaleimideI did not enhance the activation of Erk1 or Erk2 nor did basal levels of active kinase increase in the presence of the drug alone (data not shown). Therefore, the unprecedented stimulatory effects of BisI and H-89 in immortalized normal breast epithelial cells warrant further study to address the potential cross-talk between PKA and PKC in MAPK activation.

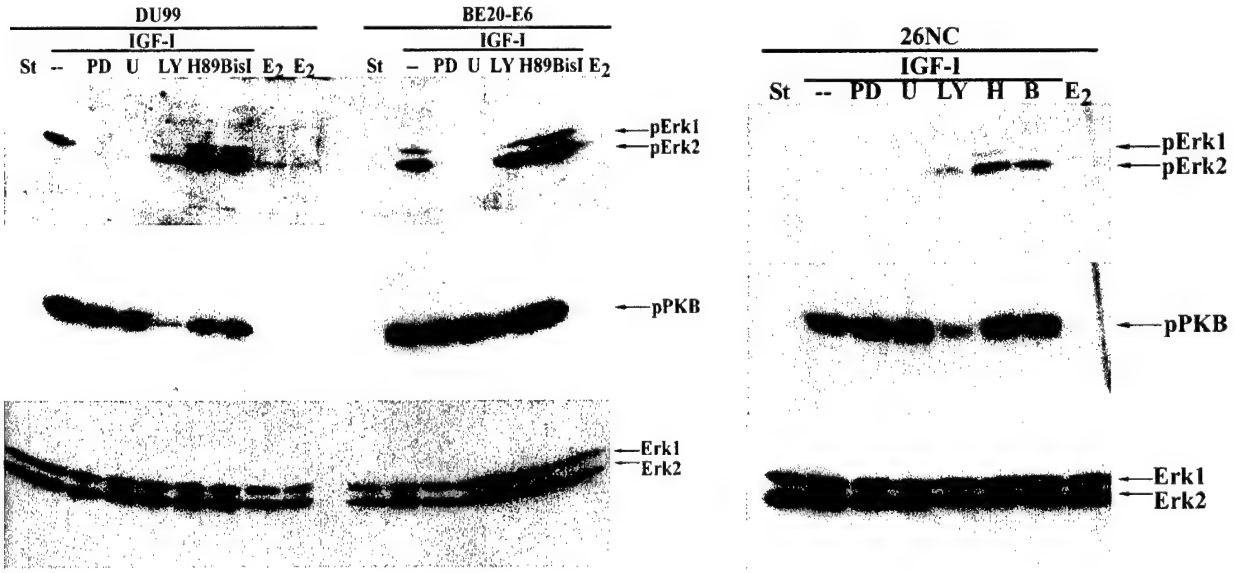


Figure 21. Effect of E_2 or IGF-I stimulation on MAPK and PKB phosphorylation status in immortalized cells. The phosphorylation status of Erk1 and Erk2 was assessed using a phospho-specific antibody and immunoblot analysis on lysates from DU99, BE20-E6, or 26NC cells treated for 5 minutes with 30 ng/ml IGF-I in the presence or absence of 50 μM PD098059 (PD), 10 μM U0126 (U), 10 μM LY294002 (LY), 2 μM ICI 182,780 (ICI), 500 nM H-89 (H), or 500 nM bisindolylmaleimideI (BisI or B). Alternatively, cells were stimulated with 2 X 10^{-8}M E_2 in the absence of any inhibitory molecules. Phosphorylated levels of PKB were assessed using a phospho-specific PKB antibody on the same cells extracts. Total Erk1 and Erk2 expression levels were detected on the same blot to control for loading variations.

The activation of MAPK in response to E_2 or IGF-I treatment was also assessed in different cultures of primary normal breast epithelial cells (Figure 22). As with the immortalized and cancerous lines, E_2 did not generate a detectable increase in active MAPK levels. IGF-I induced a significant increase in the phosphorylated form of Erk2 in all four cultures, though it did not activate Erk1. Contrary to the immortalized cells, BisI and H-89 demonstrated no effect on the activation of MAPK. Consistent with the immortalized normals and the cancerous cells, basal levels of active MAPK were inhibited in the presence of PD098059 or U0126. Therefore, in all nine of the cell lines and primary cultures examined, E_2 was unable to stimulate an increase in the levels of phosphorylated MAPK, and inhibitors of this pathway diminished the basal levels found in serum-starved cells.

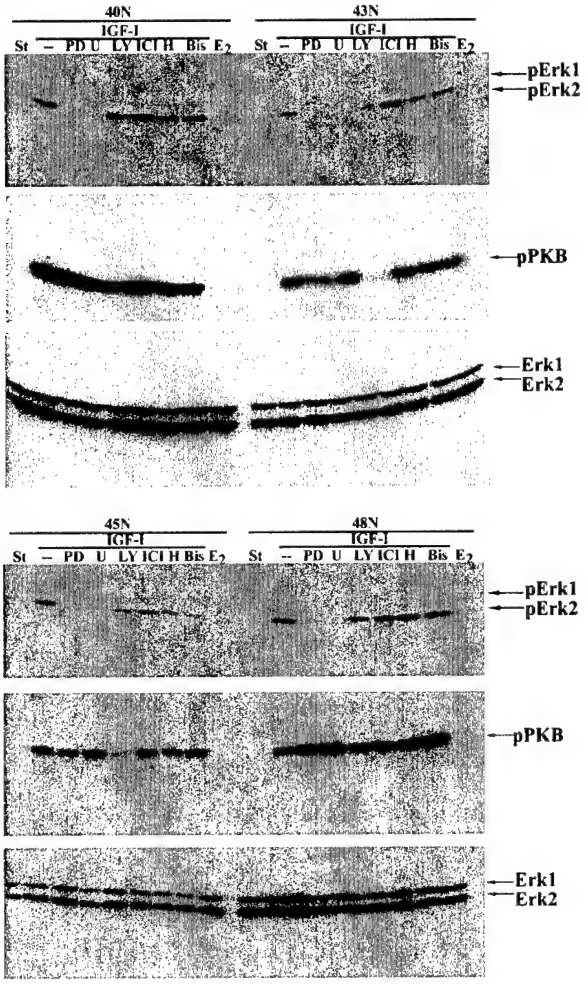


Figure 22. Effect of E_2 or IGF-I stimulation on MAPK and PKB phosphorylation status in primary normal breast epithelial cells. The phosphorylation status of Erk1, Erk2, or PKB was assessed using phospho-specific antibodies and immunoblot analysis on lysates from 40N, 43N, 45N, or 48N cells treated for 5 minutes with 2 X 10^{-8}M E_2 alone or with 30 ng/ml IGF-I in the presence or absence of 50 μM PD098059 (PD) (10 μM for 45N), 10 μM U0126 (U), 10 μM LY294002 (LY), 2 μM ICI 182,780 (ICI), 500 nM H-89, or 500 nM bisindolylmaleimideI (BisI). Phosphorylated levels of PKB were assessed using a phospho-specific PKB antibody on the same cells extracts. In order to control for loading variation, the expression levels of total Erk1 and Erk2 were detected on the same blot.

Activation of PKB in Response to Growth Factor or E_2 Stimulation in Normal Breast Epithelial Cells

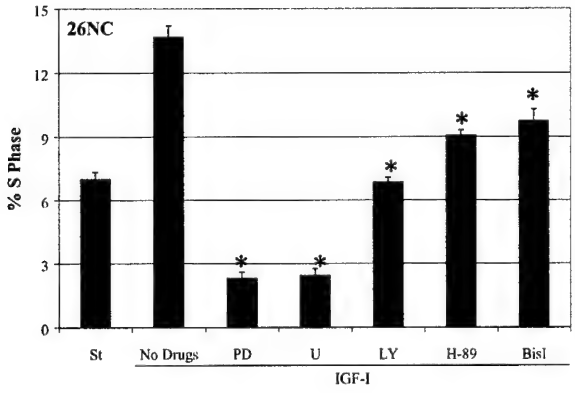
To further correlate the role of kinase cascades in the mitogenic response to IGF-I and E_2, I also examined the level of PKB phosphorylation under the same conditions. In the immortalized normal lines, E_2 did not increase the level of active PKB beyond basal levels (Figure 21). IGF-I, on the other hand, consistently induced phosphorylation, albeit at different levels in different cell lines. LY294002 prevented (or greatly reduced) the activation in each of the cell lines while the MAPK, PKA, and PKC inhibitors demonstrated little to no effect. These findings indicate that no significant differences exist between immortalized normal cells and cancerous lines in terms of the activation of PKB in response to growth factor or E_2 treatment.

Continued investigations utilizing the primary normal breast cells revealed remarkably similar findings (Figure 22). No activation of the PKB pathway occurred in response to E_2 stimulation, but in all cases, phosphorylation of PKB was stimulated by IGF-I. This activation was inhibited to varying degrees with LY294002, although it was not significantly affected by the MAPK, PKC, and PKA inhibitors as well as the pure anti-estrogen. Cumulatively, these experiments indicate that normal, immortalized, and transformed breast epithelial cells do not differ in their ability to phosphorylate PKB in response to E_2 or growth factor stimulation.

Mitogenic Response of Immortalized and Primary Normal Breast Epithelial Cells to Growth Factor or E_2 Stimulation

Having found no significant difference between the breast epithelial cells in their activation of PKB and MAPK, I wanted to determine if cell cycle profiles were also identical. The three immortalized lines as well as the four primary human mammary epithelial cell cultures were stimulated with E_2 or different growth factors for 24 hours prior to determining their rate of DNA synthesis by flow cytometry. All three of the immortalized lines (26NC, DU99, and BE20-E6) demonstrated no mitogenic response to E_2 (data not shown). However, IGF-I did stimulate cell cycle progression in each line (Figure 23). As with the cancer lines, this activity was prevented by incubation with PD098059, U0126, or LY294002. Inhibition of PKA and PKC had no effect on DU99 and BE20-E6 cells, yet diminished the maximal activity of IGF-I in 26NC cells. This indicates the potential for different cascades regulating cell cycle progression in response to the same stimulus. Overall, the immortalized cells bear a striking resemblance to the cancer lines in their requirement of MAPK and PI3-K activity for a mitogenic response.

A.



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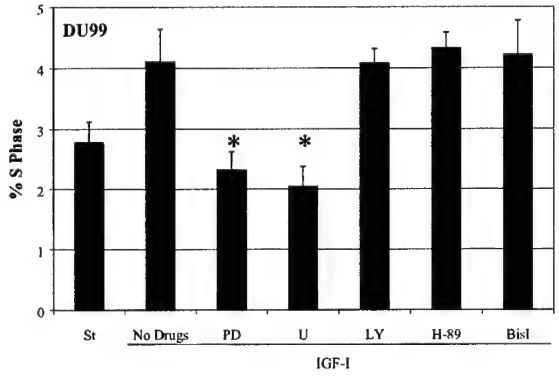

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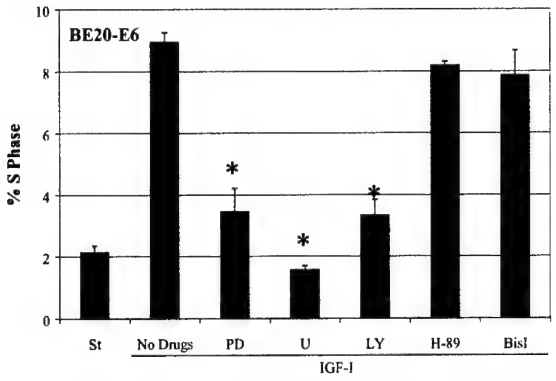

Figure 23. Effect of growth factor stimulation on cell cycle progression of immortalized breast epithelial cells. Cultures of 26NC (A), DU99 (B), and BE20-E6 (C) cells were starved for 24 hours prior to stimulation with 30 ng/ml IGF-I in the presence or absence of 50 μM PD098059 (PD) (10 μM for 45N), 10 μM U0126 (U), 10 μM LY294002 (LY), 2 μM ICI 182,780 (ICI), 500 nM H-89, or 500 nM bisindolylmaleimideI (BisI). After 24 hours of treatment cells were fixed and stained with propidium iodide, and the percentage of cells in S phase was assessed by flow cytometry after fixation and staining with propidium iodide. * denotes statistically significant decrease in IGF-I induced mitogenesis at $p \leq 0.05$. *Bars*, SD.

All four of the primary normal cell lines arrested in G1 after 24 hours of deprivation from bovine pituitary extract (Figure 24). Consistent with previous studies, E_2 did not induce a mitogenic response in any of the lines (Figure 24) (Gabelman and Emerman, 1992). Although 10 ng/ml of bFGF has been reported to stimulate the growth of separated epithelial cells, this activity only occurs in the presence of 5% serum (Gomm et al., 1997). Since these experiments were done in the absence of serum, it was therefore, not surprising to observe that bFGF also failed to induce cell cycle progression in all of the cell lines (Figure 24). EGF, which has shown mitogenic potential in normal, benign, and malignant breast tissue (Bhalla et al., 2000; Gabelman and Emerman, 1992), only stimulated proliferation in two (40N and 48N) of the four normal lines (Figure 24).

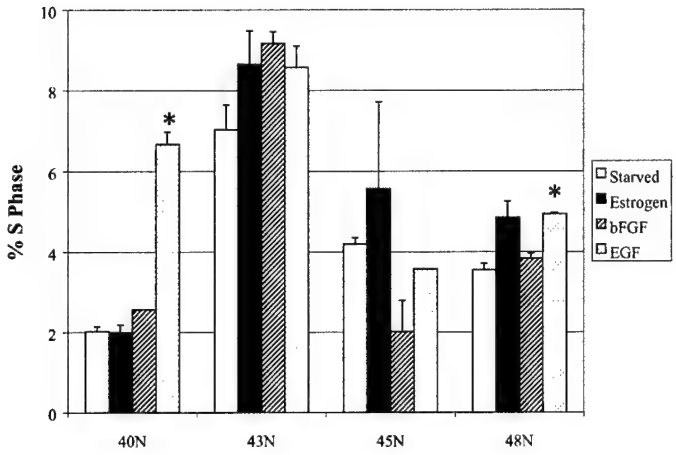

Figure 24. Effect of growth factor stimulation on cell cycle progression in primary normal breast epithelial cells. Cultures of 40N, 43N, 45N, or 48N cells were starved for 24 hours prior to stimulation with 2 X 10^{-8}M E_2, 10 ng/ml bFGF, 10 ng/ml EGF. After 24 hours of mitogenic stimulation cells were harvested, fixed, and stained with propidium iodide. The S-phase population was determined using flow cytometry. * denotes statistically significant decrease in the percentage of cells in S phase at $p \leq 0.05$. *Bars*, SD.

IGF-I, a "dominant mitogen of normal cells" in the insulin-like family (Bhalla et al., 2000), did increase the rate of DNA synthesis to a minor degree in all four of the normal lines (Figure 25). Because this induction was only slight, it was difficult to assess the effect of pharmacological inhibitors on this activity. In the 45N line, it appears as if all of the inhibitory compounds effectively prevented proliferation. In all of the lines, inhibition of MAPK activity seems to prevent a mitogenic response. The same appears to also be true for the PI3-K cascade, although sample variability prevented the identification of statistically significant changes.

Cumulatively, these studies highlight two differences between normal and malignant breast epithelial cells: (a) malignant cells are more responsive to growth factors, and (b) E_2 does not stimulate a detectable mitogenic response in normal cells. However, there is one apparent trend from these experiments: MAPK and PI3-K cascades are required for hormone or growth factor-stimulated cell cycle progression. These cascades are required in normal, immortalized, or malignant breast epithelial cells.

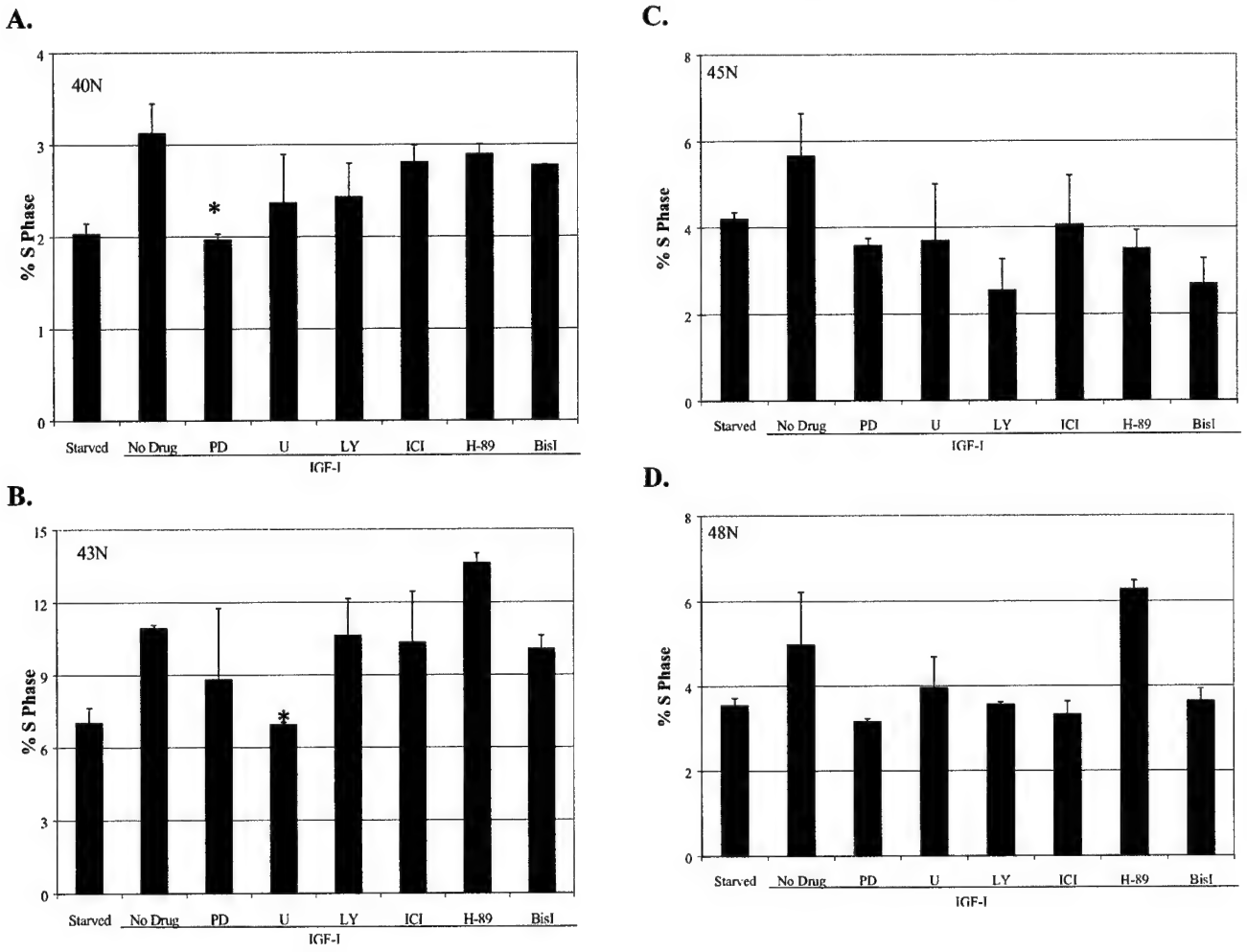

Figure 25. Effect of IGF-I treatment on cell cycle progression in primary normal breast epithelial cells. Starved cultures of 40N, 43N, 45N, or 48N cells were stimulated with 30 ng/ml IGF-I in the presence or absence of 50 μM PD098059 (PD) (10 μM for 45N), 10 μM U0126 (U), 10 μM LY294002 (LY), 2 μM ICI 182,780 (ICI), 500 nM H-89, or 500 nM bisindolylmaleimideI (BisI). After 24 hours of treatment cells were fixed and stained with propidium iodide, and the percentage of cells in S phase was assessed by flow cytometry. * denotes statistically significant decrease in IGF-I induced mitogenesis at $p \leq 0.05$. *Bars*, SD

Discussion

In Section I the requirement for MAPK and PI3-K activity for a mitogenic response to E_2 in MCF-7 cells had been demonstrated. The data presented in this section corroborate this finding and attempt to further explore the action of cytoplasmic signaling cascades inthe cell cycle progression of normal breast epithelial cells. ZR75-1 cells, an E_2-responsive cell line, proliferate when treated with either E_2 or IGF-I. Inhibitors of the MAPK and PI3-K cascades were found to prevent the proliferative action of both stimuli. Further studies demonstrated that the cell cycle block was generated prior to the G1/S transition as indicated by the lack of cdk2 activity and Rb phosphorylation status. These findings suggest that cell cycle progression stimulated by E_2 requires the action of MAPK and PI3-K, at basal levels, in a delayed-early response.

Key Research Accomplishments

- Identified the requirement for basal mitogen-activate protein kinase and phosphotidylinositol 3-kinase activity in estrogen-induced mitogenesis
- Determined the mitogen-activated protein kinase and phosphotidylinositol 3-kinase activity is essential after the expression of immediate early genes, but prior to the accumulation of cyclin D1 protein to realize the mitogenic potential of estrogen
- Demonstrated that basal levels of activity of these kinases are essential for cell cycle progression and that estrogen does not stimulate increased activity of either pathway
- Established that the minimal concentrations of estrogen sufficient to drive cell cycle progression and increase rates of transcription are identical, suggesting that these two activities are tightly associated.

Reportable Outcomes

- Sufficient data generated for completion of a doctorate in philosophy in cell and molecular biology/pathology from Duke University
- Manuscript submitted and published in the February 2000 issue of Cell Growth & Differentiation (Appendix E)
- Poster presented at the Keystone Nuclear Receptors 2000 meeting in Steamboat Springs, CO
- Manuscript submitted to the Journal of Steroid Biochemistry and Molecular Biology and currently under second review
- Postdoctoral fellowship awarded from Cindy Afshari at the National Institute of Environmental Health Sciences

Conclusions

Studies of normal and immortalized breast epithelial cells demonstrated that E_2 alone was not sufficient to stimulate proliferation. However, in cases where growth factors induced a mitogenic response, MAPK and PI3-K activity was required. This is consistent with the proposed model for growth factor action. Cumulatively, these studies indicate that MAPK is essential for cell cycle progression in

response to E_2 or growth factor stimulation of normal, immortalized, or transformed breast epithelial cells.

Previous studies have documented the signal transduction cascades that are required for growth factor-induced mitogenesis (Guan, 1994). It has been proposed that the activation of these cascades results in the activation of transcription factors. These transcription factors modulate the expression of genes that drive cell cycle progression. Surprisingly, I have identified a similar requirement in E_2-induced proliferation. It is less easy to explain the role of MAPK and PI3-K action in the mitogenic response to a hormone capable of directly affecting gene expression via its cognate receptor. Further complicating the issue is the lack of kinase activation above basal levels after E_2 treatment.

Two potential theories can explain these unprecedented findings; the first involves the action of either PI3-K or MAPK at the G1 checkpoint. PKB action can stabilize cyclin D1 protein levels. When phosphorylated on threonine 286, cyclin D1 is targeted for ubiquitin-dependent degradation (Diehl et al., 1997). This particular residue is phosphorylated through the action of GSK-3β (Diehl et al., 1998). GSK-3β is inactivated as a result of PKB action (Watts et al., 1995) and reviewed in (Coffer et al., 1998). Therefore, inhibiting the action of PI3-K would maintain the levels of active GSK-3β, thereby preventing the accumulation of cyclin D1. This mechanism implies that the cell cycle block induced by LY294002 in the presence of E_2 may be attributable to the inability of GSK3-β to be inactivated by PKB, thus blocking cyclin D1 stabilization. As demonstrated in Chapter 4, E_2 does not induce PKB activation. However, no data has been reported indicating the level of PKB activation necessary to cause cyclin D1 accumulation. Therefore, basal levels of this kinase may be sufficient.

The action of the MAPK cascade had also been implicated in proper regulation of the G1/S checkpoint. Lavoie *et al.* demonstrated that inhibition of the MAPK cascade prevented cyclin D1 accumulation as well as the expression of a reporter construct driven by the cyclin D1 promoter (Lavoie et al., 1996). Another potential role for MAPK action in the G1/S transition is regulating the assembly of cyclin D1/cdk4 complexes necessary for Rb hyperphosphorylation (Cheng et al., 1998). Therefore, the mitogenic block generated by PD098059 or U0126 in E_2-treated MCF-7 or ZR75-1 cells may be the result of preventing the expression of cyclin D1 or by inhibiting the association of cyclin D1 with cdk4 and thereby preventing the phosphorylation of Rb.

The second explanation for why cytoplasmic signaling cascades are required in the proliferative response to E_2 stimulation involves the role of kinases in the regulation of translation. Protein synthesis occurs in the cytoplasm after an mRNA has been transported out of the nucleus. Formation of the general translational machinery is an intricate process that is dependent upon the mRNA structure as well as other features of the transcript (reviewed in (Pain, 1996). The initiating event in protein synthesis is the binding of the eukaryotic initiation factor 4E (eIF4E) to the 5' cap (7-methylguanosine (5')triphospho(5')-ribonucleoside) of an mRNA (reviewed in (Rhoads, 1988) (Figure 5-9). This protein forms a trimeric complex with an RNA helicase (eIF4A) and a scaffolding protein that recruits the 40S ribosomal subunit (eIF4G) (reviewed in (Kleijn et al., 1998). This complex promotes the complete formation of the general translational machinery, and it is the regulation of complex formation and activity that represents another mechanism by which cytoplasmic signaling cascades may function in E_2-induced mitogenesis.

eIF4E is in limiting concentrations in the resting cell (Hershey, 1991), and overexpression of this protein results in transformation (Rinker-Schaeffer et al., 1993). Interestingly, eIF4E expression is 10-fold higher in breast cancer cell lines and primary tumors as compared to the levels found in normal breast epithelial cells or benign breast disease (Anthony et al., 1996; Li et al., 1997). Expression of eIF4E is regulated by the immediate early gene c-myc, and transfection studies performed with an inducible myc construct demonstrated increased eIF4E RNA and protein levels in the absence of serum (Rosenwald et al., 1993a; Rosenwald et al., 1993b). In ras transformed cells, increased levels of eIF4E are only apparent

in the presence of serum when c-myc levels are also elevated (Rosenwald et al., 1993b). Therefore, the upregulation of myc levels in response to E_2 treatment may regulate an increase in eIF4E expression.

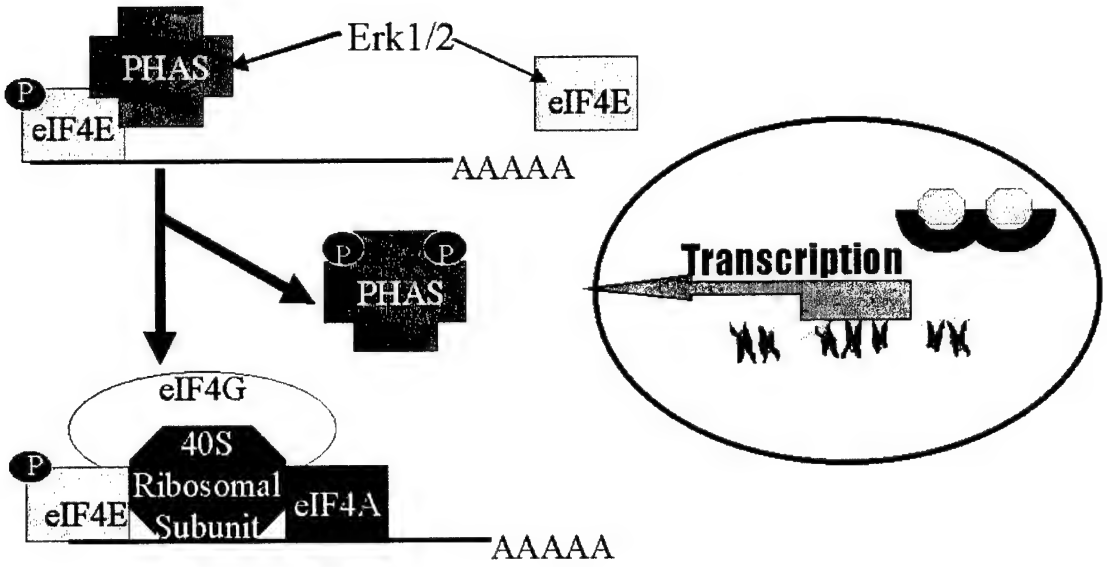

Figure 26. Initiation of protein synthesis. The binding of eIF4E is the initiating event of translation. Phosphorylation of eIF4E increases the binding affinity for the 5'cap of the RNA transcript. Phosphorylation of the negative regulator of eIF4E activity, PHAS-I, results in dissociation of this protein and enables complex formation of the general translational machinery. eIF4E is a substrate for MAPK and PHAS-I is a substrate for PKB as well as MAPK. The basal activity of these kinases may be required for the initiation of protein synthesis of key genes involved in cell cycle progression. The mRNA for these genes is likely to be increased as a result of ER-mediated transcription (as illustrated in the nucleus). Therefore, inhibitors of MAPK and PI3-K activity may prevent E_2-induced mitogenesis by blocking the synthesis of protein products necessary of proliferation.

eIF4E is believed to modulate the translation of genes with long untranslated regions that are G + C rich. This is presumed to occur via the association of eIF4E with an RNA helicase that is capable of relieving the secondary structure and enabling formation of the general translational machinery (reviewed in (Rhoads, 1988; Sonenberg, 1993). The untranslated region of cyclin D1 is longer than average (170 nucleotides compared to 50 to 100 nucleotides for actin and GAPDH). Cyclin D1 also has a high G + C content (~70%). Numerous studies have demonstrated that expression of eIF4E results in increased nucleocytoplasmic shuttling of cyclin D1 transcript, increased numbers of polysomes associated with the cyclin D1 message, and ultimately an increase in the protein levels of cyclin D1 (Rosenwald et al., 1995; Rosenwald et al., 1993a; Rousseau et al., 1996b). Therefore, the increase of cyclin D1 levels in E_2-treated cells may be the result of eIF4E activity resulting from c-myc expression.

The activity of eIF4E protein is modulated in one of two ways. eIF4E activity can be inhibited by associating with a negative regulator, PHAS-I (Phosphorylated Heat-and Acid-Stable protein, Insulin-

stimulated) (reviewed in (Lawrence and Abraham, 1997). When PHAS-I is associated with eIF4E, it prevents the initiation of translation by competing for the same binding site as eIF4G (Haghighat et al., 1995). Since PHAS-I antagonizes the action of eIF4E, it was not surprising that overexpression of PHAS-I in eIF4E transformed cells significantly reverts the phenotype to an untransformed state (Rousseau et al., 1996a). PHAS-I is a phosphoprotein (Pause et al., 1994), and when in its phosphorylated state, it dissociates from eIF4E, enabling complex formation with eIF4G and subsequent translation. Recent studies have demonstrated that PHAS-I is a substrate for both MAPK as well as PKB (Gingras et al., 1998; Haystead et al., 1994). Therefore, the mitogenic block observed in E_2-stimulated cells in the presence of PI3-K and MAPK inhibitors may be attributable to decreased synthesis of protein products from genes that are necessary for cell cycle progression (Figure 26).

The activity of eIF4E can also be regulated directly by post-translational modifications. Phosphorylated eIF4E demonstrates a higher affinity than the unphosphorylated form for the mRNA cap (Minich et al., 1994). This facilitates its participation in protein synthesis. Phosphorylation of eIF4E in NIH 3T3 cells stimulated with serum was inhibited by PD098059, the pharmacological inhibitor of MAPK activity (Morley and McKendrick, 1997). Therefore, an alternative explanation for the cell cycle arrest in the presence of MAPK inhibitors in E_2-treated cells may be decreased protein synthesis resulting from a lower affinity of eIF4E for mRNA caps due to the absence of basal levels of active MAPK (Figure 5-9).

In summary, this work further characterized the role of cytoplasmic signaling cascades in the molecular response of breast epithelial cells to E_2 stimulation. Though the MAPK and PI3-K cascades do not appear to be activated beyond basal levels in response to E_2 treatment, the activity of both is required for cell cycle progression. The action of these cascades appears to occur after the increase in immediate early gene transcripts and prior to the G1 checkpoint. Whether the activity of these kinases are required at the level of cyclin D1 stabilization, translation regulation, or some other critical point in the G1 phase of the cell cycle will have to be determined by additional experimentation. Elucidating the exact molecular mechanism responsible for E_2-induced proliferation may result in the identification of additional targets for drug discovery screens to prevent the continued growth of hormone responsive breast cancers.

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Appendix A

Pharmacological Inhibitor Reference Chart

Drug Name	Abbv	Mechanism of Inhibition	Ref
PD098059	PD	Inhibits the activation of MEK1 by an allosteric mechanism	(Alessi et al., 1995; Dudley et al., 1995)
U0126	U	Inhibits the activity of MEK1/MEK2 by an allosteric mechanism	(Favata et al., 1998)
LY294002	LY	Competitive inhibitor of the ATP-binding site of PI3-K	(Vlahos et al., 1994)
Bisindolyl-maleimideI	BisI	Competitive inhibitor of the ATP-binding site of PKA	(Martiny-Baron et al., 1993; Toullec et al., 1991)
H-89, Dihydochloride	H-89	Competitive inhibitor of the ATP-binding site of PKA	(Chijiwa et al., 1990)
ICI 182,780	ICI	High affinity ER ligand that results in receptor degradation	(Pink and Jordan, 1996; Wakeling and Bowler, 1992; Wakeling et al., 1991)

Appendix B

Dominant Negative Constructs and Antisense Oligonucleotides

A genetic approach was taken to substantiate the data generated using pharmacological inhibitors of the MAPK cascade. MCF-7 cells were transiently transfected with one of the following: (a) control plasmid (pcDNA3.1), (b) wild-type MEK1, (c) dominant negative MEK1 (K97A), (d) wild-type Erk1, (e) dominant negative Erk1 (membrane-targeted due to a CAAX motif), (f) wild-type Erk2, (g) dominant negative Erk2 (membrane-targeted due to a CAAX motif), (h) antisense MAPK phosphorothioated oligonucleotide, or (i) a scrambled antisense control (Hochholdinger et al., 1999; Robinson et al., 1996; Sale et al., 1995; Seger et al., 1994). After transfection with either the FuGENE 6 or Lipofectin transfection reagents, cells were serum-starved to arrest the cells in G0/G1 and then stimulated for 24 hours with 2 X 10^{-8}M E_2. After stimulation, the percentage of transfected cells in S phase was determined by assaying the relative levels of propidium iodide staining of GFP-positive cells. This analysis revealed that transfected MCF-7 cells no longer serum-starve as effectively; therefore, the effect of E_2 stimulation could not be ascertained (data not shown).

Flow cytometric analysis revealed that the cell cycle profiles of serum-starved cells varied regardless of the transfection reagent used. Figure B-2 illustrates the differences in a profile of serum-starved MCF-7 cells versus comparably treated cells that had been transfected with pcDNA3.1 control using either FuGENE6 or Lipofectin. There is not only an increased percentage of cells in S phase in both transfection conditions, but also a diminished G2/M peak. These results were consistently observed with different control plasmids and different batches of both transfection reagents. These findings suggested that transiently transfecting the MCF-7 cell line maintained in our laboratory generated artifactual effects on cell cycle progression. Therefore, this was not an effective means to verify the pharmacological data. In order to avoid generating artifactual data resulting from the non-specific effects of an individual drug, two inhibitors for each pathway (PD098059 and U0126 for MAPK; LY294002 and wortmannin for PI3-K) were consistently used. Furthermore, the following characterization of PI3-K and MAPK activity in E_2-induced mitogenesis was also analyzed in another ER+ breast epithelial cell line, ZR75-1 (Section III).

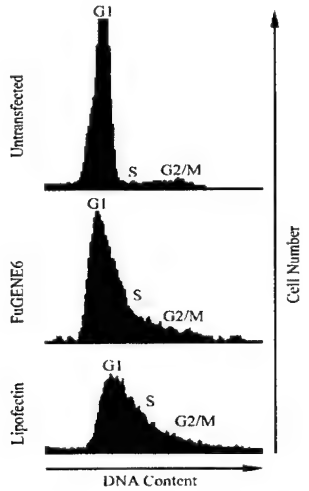

Figure 27. Cell cycle profiles of serum-starved MCF-7 cells transfected with FuGENE6 or Lipofectin. MCF-7 cells were transfected, using either the FuGENE6 or Lipofectin reagent, with a control plasmid (pcDNA) and a green fluorescent protein expression construct (pEGFP-NI) as a marker for transfection efficiency. Cells were fixed after 24 hours of serum-starvation and the cell cycle profile was assessed by staining with propidium iodide and analyzing the DNA content by flow cytometry of GFP^+ cells.

Appendix C
Cell Line Reference Chart

Cell Line Name	Origin
MCF-7	Mammary adenocarcinoma cell line
ZR75-1	Mammary adenocarcinoma cell line
Ishikawa	Uterine adenocarcinoma cell line
26NC	Normal breast epithelial cell immortalized with DMBA
DU99	Normal breast epithelial cell immortalized with the catalytic subunit of telomerase
BE20-E6	Normal breast epithelial cell immortalized with the human papilloma virus E6 gene
40N	Normal breast epithelial cell generated from tissue obtained from a reduction mammoplasty
43N	Normal breast epithelial cell generated from tissue obtained from a reduction mammoplasty
45N	Normal breast epithelial cell generated from tissue obtained from a reduction mammoplasty
48N	Normal breast epithelial cell generated from tissue obtained from a reduction mammoplasty

Appendix D
Publications, Meeting Abstracts, and List of Personnel

Publications

Lobenhofer, E.K., Huper, G., Iglehart, J.D., and Mark, J.R. (2000). Inhibition of mitogen-activated protein kinase and phosphatidylinositol 3-kinase activity in MCF-7 cells prevents estrogen-induced mitogenesis, Cell Growth Differ *11*, 99-110.

Lobenhofer, E.K. and Marks, J.R. Estrogen-Induced Mitogenesis of MCF-7 Cells Does Not Require the Induction of Mitogen-Activated Protein Kinase Activity, J Steroid Biochem Mol Biol *Submitted.*

Meeting Abstracts

Lobenhofer, E.K. and J.R. Marks. (2000) The involvement of cytoplasmic signaling cascades in estrogen-induced mitogenesis. Duke University Medical Center: Department of Pathology Research Retreat. Durham, North Carolina.

Lobenhofer, E.K. and J.R. Marks. (2000) Inhibition of cytoplasmic kinase activity prevents estrogen-induced mitogenesis. Era of Hope: Department of Defense Breast Cancer Research Program Meeting. Atlanta, Georgia.

Lobenhofer, E.K. and J.R. Marks. (2000) The involvement of cytoplasmic signaling cascades in estrogen-induced mitogenesis. Keystone Symposia: Nuclear Receptors 2000, Steamboat Springs, Colorado.

List of Personnel

Lobenhofer, E.K.

Vol. 11, 99–110, February 2000

Inhibition of Mitogen-activated Protein Kinase and Phosphatidylinositol 3-Kinase Activity in MCF-7 Cells Prevents Estrogen-induced Mitogenesis[1]

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Abstract

Estrogen acts to promote DNA synthesis in the MCF-7 human breast cancer cell line via its interaction with high levels of estrogen receptor. The primary mode of estrogen action has been considered to be through transcriptional activation of genes containing estrogen response elements, including the immediate early genes c-*myc* and *fos*. Recent reports have indicated that estrogen, acting through the estrogen receptor, is capable of inducing the mitogen-activated protein kinase (MAPK) cytoplasmic signaling cascade. In this study, specific small molecule inhibitors of MAPK and phosphatidylinositol 3-kinase activity were used to determine the influence of these cascades on estrogen-mediated mitogenesis. Phosphatidylinositol 3-kinase inhibitors, LY294002 and wortmannin, as well as inhibitors of MAPK kinase-1, PD098059 and U0126, decreased the fraction of cells entering DNA synthesis after treatment with 17β-estradiol. These compounds did not inhibit expression of myc or fos. However, the drugs did prevent the accumulation of cyclin D1 and hyperphosphorylated retinoblastoma protein, indicating that the block occurred at, or prior to, this point in the cell cycle. Although these compounds were effective in preventing estrogen-mediated mitogenesis, the downstream kinases extracellular signal-regulated kinase 1, extracellular signal-regulated kinase 2, and protein kinase B were not activated over basal levels by estrogen treatment. These studies suggest that estrogen initiates mitogenesis by inducing the transcription of immediate early genes, but cytoplasmic signaling pathways play an important role in the control of subsequent events in the cell cycle.

Introduction

Estrogen can act as an effective mitogen in cells that express the ER[3] both in cell culture and *in vivo* (reviewed in Ref. 1). E_2, the most prevalent estrogen produced by the ovaries, bound to ER results in receptor translocation to the nucleus and dimerization. The ligand-bound ER dimer can interact with DNA at palindromic EREs, resulting in modulation of transcriptional activity. Antiestrogen treatment of cells that depend upon estrogen for their growth blocks this transcriptional activation and results in a G_0-G_1 cell cycle arrest (2). A number of genes contain putative EREs including the immediate-early genes, c-*myc* and c-*fos* (3, 4). Therefore, it has been postulated that the mechanism by which estrogen mediates mitogenesis is via transcriptional activation of these and other key cell cycle regulatory genes.

After immediate-early gene synthesis, the cell cycle converges on the expression and activity of the cyclins and CDKs, particularly the D-type cyclins. Estrogen treatment of growth-arrested MCF-7 cells, a cancerous breast epithelial cell line that overexpresses ER, results in increased expression of cyclin D1 (5). Concurrently, the activity of cyclin D1 binding partners, CDK4 and CDK6, increases (6, 7). Formation of active cyclin D1-CDK4 and cyclin D1-CDK6 holoenzymes serves two functions: (*a*) redistribution of CDK inhibitors (such as $p27^{Kip1}$ and $p21^{Cip1}$) away from cyclin E, facilitating the formation of active cyclin E-CDK2 complexes; and (*b*) phosphorylation of the Rb protein, which is also phosphorylated by cyclin E-CDK2 (8–10).

The subcellular localization of ER and its role as a transcription factor led to the assumption that estrogen action occurs primarily in the nucleus. However, several recent reports have shown that elements of cytoplasmic signaling cascades may also be estrogen responsive. In cells expressing ER, the MAPK family members Erk1 and Erk2 are activated within 5 min in response to E_2 treatment (11–14). Furthermore, cell membrane-impermeable estrogen (estradiol conjugated to BSA) stimulates MAPK activity (15). A possible explanation for this phenomenon comes from several studies that have reported the existence of plasma membrane-associated ER (16–19). Activation of MAPK raises the

Received 6/17/99; revised 12/20/99; accepted 12/21/99.
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[1] This work was supported by Predoctoral Training Grant DAMD17-97-1-7224 (to E. K. L.) from the Department of Defense and the Duke University Medical Center Specialized Programs of Research Excellence in Breast Cancer Grants PHS CA68438 and PHS CA73802.
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[3] The abbreviations used are: ER, estrogen receptor; E_2, 17β-estradiol; ERE, estrogen response element; CDK, cyclin-dependent kinase; Rb, retinoblastoma; MAPK, mitogen-activated protein kinase; Erk, extracellular signal-regulated kinase; MEK, MAPK kinase; PKA, protein kinase A; PKB, protein kinase B; PKC, protein kinase C; PI3-K, phosphatidylinositol 3-kinase; EGF, epidermal growth factor; IGF, insulin-like growth factor; GSK, glycogen synthase kinase; FBS, fetal bovine serum.

possibility that some or all of the mitogenic activity of estrogen may be mediated through this pathway.

This theory was also supported by a study demonstrating that a MAPK inhibitor (PD098059) prevented estrogen-responsive proliferation in cardiac fibroblasts (20). Furthermore, estrogen was able to induce mitogenesis in NIH-3T3 cells transiently transfected with transcriptionally inactive ER, presumably because of the ability of E_2 to activate MAPK (21). Cumulatively, these studies suggest that MAPK activation may be a requirement for estrogen-induced cell cycle progression.

Estrogen and its receptor may also interact with other cytoplasmic signaling components. In endometrial fibroblasts as well as selected malignant cell lines, E_2 stimulates the activity of PKC (22). Furthermore, in MCF-7 cells, synthesis of phosphatidylinositol and activation of phosphatidylinositol kinases can occur in response to E_2 treatment (23). ER itself can also act as a substrate for PKA. Serine-236 can be phosphorylated *in vitro* by PKA, which may be important in receptor dimerization (24). Collectively, these reports suggest that cytoplasmic factors may be important intermediates in estrogen action.

In previous work, we found that MCF-7 cells grown in medium alone (no serum or added growth factors) proliferate in response to estrogen (25). This system provides a model for studying the mitogenic effects of E_2 in the absence of other exogenously added stimulatory molecules. In the current study, we have used a series of pharmacological compounds that specifically inhibit signaling intermediates that have been implicated in estrogenic action. Our results show that MAPK and PI3-K, but not PKA or PKC, activity are required for cell cycle progression in response to E_2 stimulation. The cell cycle block caused by MAPK and PI3-K inhibitors occurs after the expression of immediate-early genes and prior to the induction of cyclin D1 expression, resulting in G_1 arrest. Additional experiments show that E_2 does not significantly activate MAPK or PKB, a downstream target of PI3-K, suggesting that either basal levels or delayed induction of these kinases are required for cell cycle progression in MCF-7 cells.

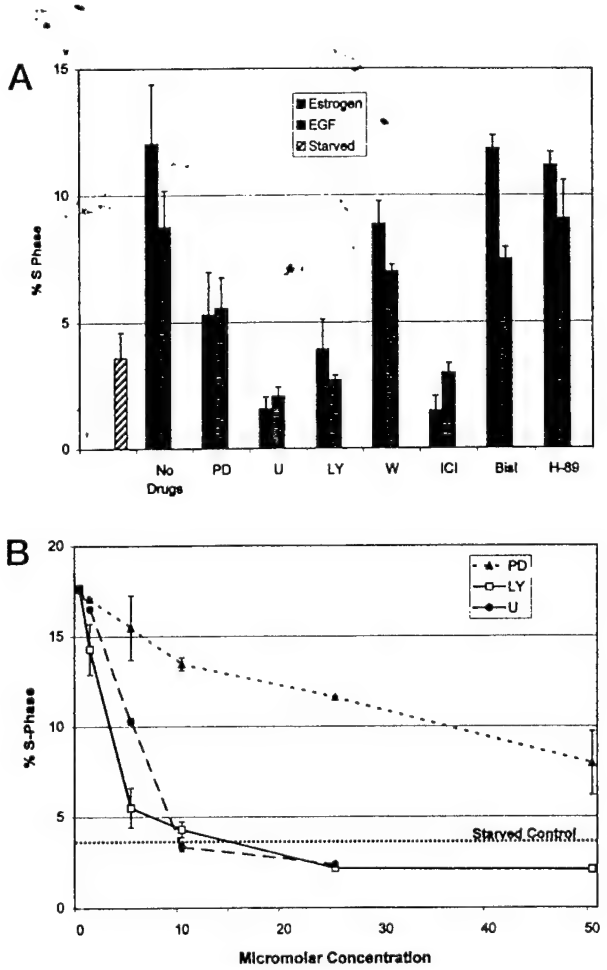

Fig. 1. Effect of cytoplasmic signaling inhibitors on E_2-induced mitogenesis. *A*, percentage of MCF-7 cells in S phase after treatment with 2×10^{-8} M E_2 or 10 ng/ml EGF for 24 h in the presence or absence of 50 μM PD098059 (*PD*), 25 μM U0126 (*U*), 5 μM LY294002 (*LY*), 100 nM wortmannin (*W*), 2 μM ICI 182,780 (*ICI*), 750 nM bisindolylmaleimide I (*Bisl*), or 500 nM H-89. Compounds such as ICI 182,780, which inhibit MCF-7 cells by generating a potent cell cycle block, will exhibit lower rates of DNA synthesis than the asynchronous serum-starved control. The data represent an average of results obtained from three experiments, each performed in triplicate. *Bars*, SD. *B*, estrogen-stimulated MCF-7 cells were treated with variable concentrations of PD, LY, and U for 24 h, and the rate of DNA synthesis was measured. *Bars*, SD.

Results

Cytoplasmic Signaling Inhibitors Prevent Estrogen-induced Mitogenesis. The breast cancer epithelial cell line MCF-7 expresses high levels of ERα. When these cells are serum-starved in phenol red-free media for 24 h, the largest fraction of cells accumulates and arrests early in the G_1 stage of the cell cycle. Treatment of starved cells with estrogen alone (2×10^{-8} M E_2) stimulated cell cycle progression and resulted in a 3–4-fold increase in the number of cells undergoing DNA synthesis, a rate that is comparable or greater than the mitogenic effect of EGF (Fig. 1*A*).

Several signaling cascades have been implicated in E_2 function. To assess the importance of these pathways on E_2-induced mitogenesis, we examined changes in the rate of DNA synthesis as a result of exposure to specific inhibitors. Serum-starved cells were stimulated with E_2 in the presence of inhibitors of MEK, the upstream activator of MAPKs (50 μM PD098059 or 25 μM U0126), PI3-K (5 μM LY294002 or 100 nM wortmannin), PKA (500 nM H-89), PKC (750 nM bisindolylmaleimide I), or with an ER antagonist (2 μM ICI 182,780). These concentrations were chosen based upon published values demonstrated to inhibit each of the kinases effectively without causing nonspecific toxicity (26–29). Cell cycle distribution was measured by flow cytometry after 24 h of treatment (results from three representative experiments are averaged in Fig. 1*A*). The antiestrogen, as expected, effectively blocked E_2- induced DNA synthesis, reducing the percentage of cells in S phase to below the levels in starved cells. Neither the PKA or PKC inhibitors (H-89 and Bisl) had any significant effect. However, the MEK1 inhibitor, PD098059, reduced the rate of DNA synthesis by >50%; whereas the MEK1/MEK2 inhibitor, U0126 (U), decreased the rate of proliferation to below the level observed in starved cells. Inhibi-

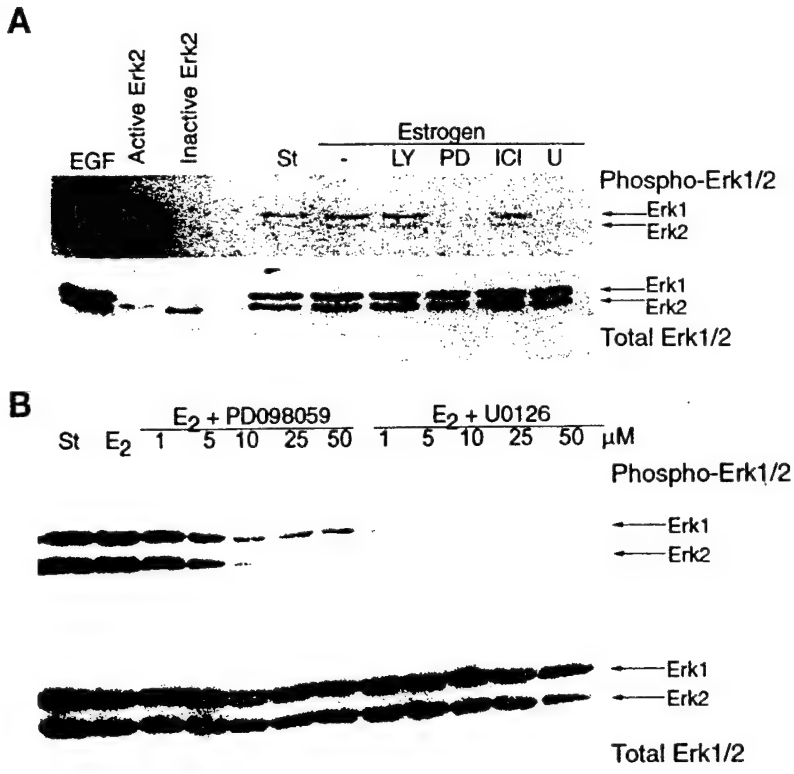

Fig. 2. Effect of E_2 stimulation on MAPK phosphorylation status. *A*, the phosphorylation status of Erk1 and Erk2 was assessed using a phosphospecific antibody and immunoblot analysis on lysates from cells treated with 10 ng/ml EGF or 2×10^{-8} M E_2 in the presence or absence of 5 μM LY294002 (*LY*), 50 μM PD098059 (*PD*), 2 μM ICI 182,780 (*ICI*), or 25 μM U0126 (*U*). Levels of total Erk1 and Erk2 were detected on the same blot to control for loading variations. *B*, phosphorylated and total levels of Erk1 and Erk2 were assessed using the technique described above for cells treated with 2×10^{-8} M E_2 in the presence of 1, 5, 10, 25, or 50 μM PD098059 or U0126.

tion of the PI3-K cascade with LY294002 was almost as effective as the ICI antiestrogen in blocking E_2-induced S phase. However, wortmannin was not as potent an inhibitor. These data suggest that the activities of at least two cytoplasmic signaling molecules (MEK1 and PI3-K) are critical for the mitogenic effect of estrogen.

To compare the effects of these inhibitors on another ligand that is mitogenic in MCF-7 cells, we repeated the experiments on this breast cancer cell line stimulated with EGF (30). As reported previously (31), the ICI antiestrogen inhibited EGF-induced mitogenesis. Furthermore, EGF-stimulated DNA synthesis was inhibited by the same compounds to virtually the same degree as was seen with E_2 treatment (Fig. 1*A*).

To determine the efficacy of these drugs in inhibiting estrogen-induced proliferation, we measured S-phase progression in the presence of a range of inhibitor concentrations (1–50 μM). At the lowest concentration for each drug, minimal effects, if any, were observed (Fig. 1*B*). However, in the presence of 5 μM LY294002, the percentage of cells in S phase was maintained at or near basal levels, a finding that is supported by the published IC_{50} (1.4 μM) of LY294002 for abolishment of PI3-K activity (26). Similar kinetics of inhibition were noted with the MEK1/MEK2 inhibitor, U0126. PD098059 only weakly inhibits the activation of MEK2 and has a much greater IC_{50} for the inhibition of MEK1 as compared with U0126 (29). Therefore, it was not surprising to note that PD098059 did not have as pronounced an inhibitory effect on estrogen-induced mitogenesis.

Effect of Estrogen on the Activation of the MAPK Cascade. Inhibition of the PI3-K and MAPK cascades prevented efficient estrogen-induced cell cycle entry in MCF-7 cells. Several recent reports have demonstrated that the MAPK pathway is stimulated by estrogen in an ER-dependent fashion (12, 13, 15, 21, 32). The dual specificity kinases, MEK1 and MEK2, phosphorylate a threonine and a tyrosine residue in the regulatory sites of Erk1 and Erk2, resulting in the activation of these MAPKs (reviewed in Ref. 33). We performed a series of experiments to determine whether Erk1 and/or Erk2 were activated by E_2 under our conditions (Fig. 2*A*). The dually phosphorylated forms of Erk1 and Erk2 were specifically recognized on the immunoblot using a phosphospecific monoclonal antibody, whereas total Erk1 and Erk2 was detected using a separate antibody after stripping and reprobing the same blot. In this case, we measured activation 5 min after treatment with either EGF, a known inducer of MAPK activity, or E_2. Compared with the untreated control (starved cells), 10 ng/ml EGF induced a dramatic increase in phosphorylated Erk1 and Erk2. Under the same conditions, 2×10^{-8} M E_2 had little or no effect on the levels of activated Erk1 or Erk2 (Fig. 2*A*). A number of repetitions of this experiment failed to show more than a 1.5-fold induction at any time point (from 1 to 20 min after treatment) or at any cell density. Neither ICI 182,780 nor LY294002 had any effect on active MAPK levels in E_2-stimulated cells (Fig. 2*A*). Although estrogen failed to activate MAPK, the MAPK inhibitors (PD098059 and U0126) effectively reduced the basal levels of phospho-Erk1 and -Erk2 (Fig. 2*A*).

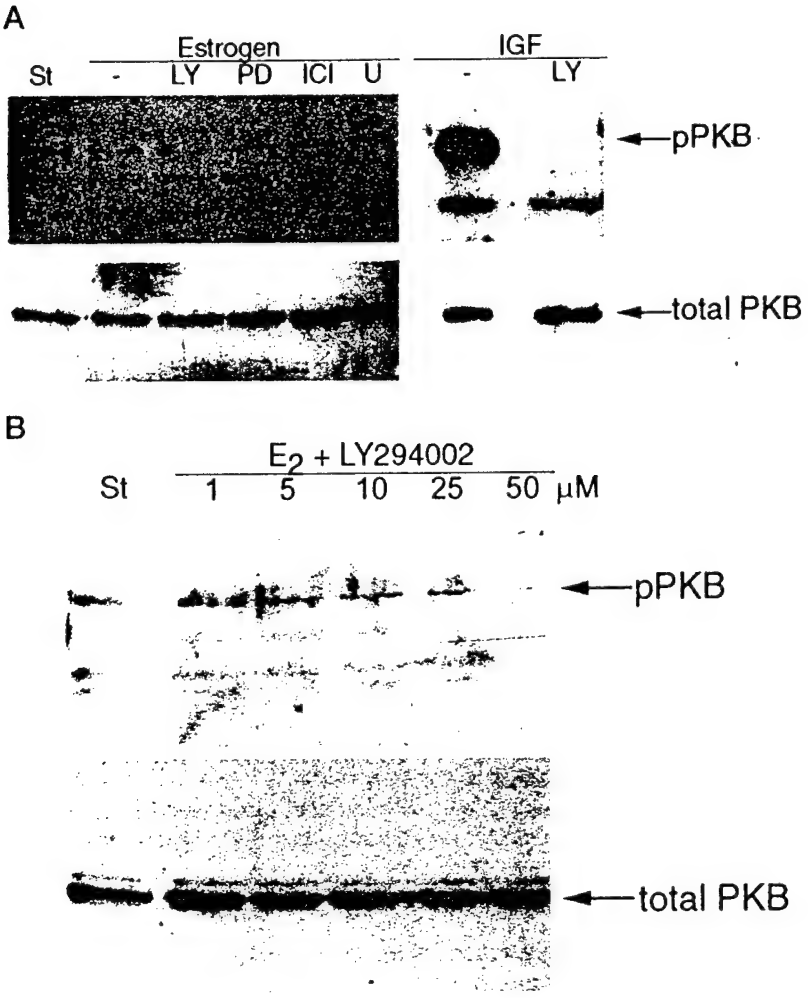

Fig. 3. Effect of E_2 stimulation on PKB phosphorylation status. *A*, phosphorylation of PKB was determined by Western analysis using a phosphospecific antibody on MCF-7 cell extracts from cells stimulated with 30 ng/ml IGF-I in the presence or absence of 5 μM LY294002 (*LY*) or with 2×10^{-8} M E_2 in the presence or absence of 5 μM LY, 50 μM PD098059 (*PD*), 2 μM ICI 182,780 (*ICI*), or 25 μM U0126. The detection of total levels of PKB on the same blot controlled for loading discrepancies. *B*, phosphorylated and total levels of PKB were assessed using the technique described above for cells treated with 2×10^{-8} M E_2 in the presence of 1, 5, 10, 25, or 50 μM LY294002.

The ability of U0126 and PD098059 to inhibit basal levels of activated MAPKs as well as cell cycle progression prompted us to examine whether a correlation exists between these two activities. Using the same antibodies described above, MCF-7 cells were stimulated with estrogen for 5 min in the presence of variable concentrations of PD098059 or U0126 (Fig. 2*B*). Estrogen had no effect on Erk1 or Erk2 levels, but increasing concentrations of these compounds inhibited phosphorylation of these kinases. A clear reduction in phospho-Erk1 and -Erk2 was noted with as little as 1 μM U0126 compared with starved or E_2-treated cells. Peak inhibition was achieved between 5 and 10 μM. The PD098059 compound also reduced the levels of active Erk1/Erk2 with maximum inhibition reached at ~10 μM. Even at the highest concentration tested (50 μM), PD098059 was unable to completely inhibit phospho-Erk1/Erk2 levels. These data are consistent with relative S-phase inhibition observed with these two compounds and suggest that maintenance of basal levels of phospho-Erk1/Erk2 may be important in E_2-mediated mitogenesis.

Effect of Estrogen on the Activation of the PI3-K Cascade. PKB is a primary downstream target of PI3-K activation and has been implicated in cyclin D1 protein stabilization (34). Treatment with estrogen had little, if any, effect on phosphorylated (active) PKB levels compared with stimulation with 30 ng/ml IGF-I, a known activator of PI3-K in MCF-7 cells (Fig. 3*A*; Ref. 35). Neither PD098059, U0126, nor ICI 182,780 affected the phosphorylation of PKB; however, the PI3-K drug, LY294002, was a potent inhibitor of PKB activation in response to IGF.

The effect of LY294002 on the basal levels of phosphorylated PKB was also investigated. Only at the highest concentration (25 μM) was PKB significantly inhibited relative to starved or E_2-treated cells (Fig. 3*B*). However, near-maximal inhibition of S-phase was achieved using 5 μM (Fig. 1*B*). As measured by phosphorylation of PKB, inhibition of the basal activity of PI3-K does not appear to correlate with suppression of E_2-induced growth.

Toxicity and Reversibility of PI3-K and MAPK Inhibitor Molecules. We next examined toxicity and reversibility of the pharmacological inhibitors of MAPK, PI3-K, and ER. Cell viability was measured by dye exclusion after 24 h of drug treatment in the presence of 2×10^{-8} M E_2 (Fig. 4). A slight decline in viability was observed in cultures treated with the MAPK inhibitors PD098059 and U0126 and with the estrogen antagonist ICI 182,780. We observed no increase in the

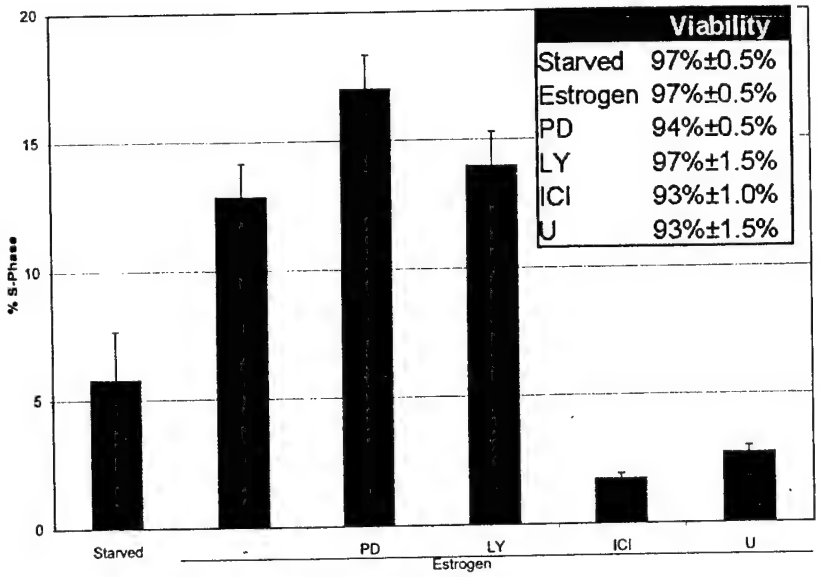

Fig. 4. Reversibility of cytoplasmic signaling inhibitors and their effect on cell viability. Serum-starved MCF-7 cells were treated with 50 μM PD098059 (*PD*), 5 μM LY294002 (*LY*), 2 μM ICI 182,780 (*ICI*), or 25 μM U0126 (*U*) for 24 h. Cells were then washed three times with starvation media prior to stimulation with 2×10^{-8} M E_2 in the absence of any inhibitory compounds for 24 h. The rate of DNA synthesis was then measured by propidium iodide staining and analysis by FACS. *Inset*, after 24 h of serum starvation, MCF-7 cells were stimulated with 2×10^{-8} M E_2 in the presence or absence of the pharmacological compounds listed above (using the same dosage). Twenty-four h after treatment, cell viability was assessed by staining with erythrosin B and determining the percentage of viable cells. *Bars*, SD.

apoptotic fraction with these drugs, as measured by flow cytometry (data not shown). Next, we measured the reversibility of the growth-inhibitory effects of each drug by a washout experiment. MCF-7 cells were treated with the small molecule inhibitors for 24 h in the absence of any mitogen prior to washing three times with PBS and stimulating with E_2 (Fig. 4). Cell cycle analysis showed that cells treated with LY294002 and PD098059 could respond mitogenically to estrogen stimulation after the drugs had been removed. However, inhibition by U0126 and ICI 182,780 could not be reversed in this time frame. Because this did not appear to be related to toxicity, it appears that these drugs exert a long-lasting effect on their targets. The ICI antiestrogen acts by inducing ER degradation; therefore, a long-lasting effect on ER response is not surprising (36).

Inhibitor Effects on ER-mediated Transcription. The most well-understood mode of estrogen action is the transcriptional activation of genes containing EREs. Among the genes containing putative EREs are several key cell cycle regulatory genes, including the immediate-early genes c-*myc* and c-*fos* (3, 4). If the PI3-K and MAPK inhibitors affected transcriptional regulation by the ER, they could stop cell cycle progression by preventing the induction of these genes. To determine whether the effects of signaling inhibitors on E_2-stimulated mitogenesis were attributable to interference with ER-mediated transcriptional activity, MCF-7 cells were transfected with a luciferase gene under the control of either a naturally occurring (vitellogenin, TK-ERE) or artificial E_2-responsive promoter (3X-ERE), and cell extracts were harvested 24 h later. In the absence of drugs, E_2 induced a 5–6-fold increase in luciferase activity as opposed to the near basal levels seen in the presence of the ER antagonist ICI 182,780 (Fig. 5*A*). Blocking PKA or PKC activation had no impact on ER-mediated transcription, whereas the MEK1/2 inhibitor U0126 was as effective as the ICI compound at preventing ER-mediated transcription. Transcription declined ~50% in the presence of the MEK1 inhibitor PD098059 and with the PI3-K inhibitor LY294002.

To examine the impact of cytoplasmic signaling cascades on earlier transcriptional events, this experiment was repeated with cell extracts harvested at shorter intervals after estrogen addition. Samples taken after 6 or 9 h displayed the same kinetics of induction and inhibition as the 24-h time point (data not shown). Attempts at detecting even earlier effects (2 h) using this reporter gene assay did not yield interpretable results because E_2 alone failed to induce detectable luciferase activity.

The specificity of drug effects on ER-mediated transcription was examined by studying their impact on the transcriptional activity of another steroid hormone, progesterone. MCF-7 cells were transiently transfected with a human progesterone receptor expression vector and a luciferase gene with a progesterone-responsive promoter. Addition of 10^{-7} M progesterone for 24 h stimulated a 5-fold increase in luciferase activity, an activity that was not inhibited in the presence of LY294002 and ICI 182,780 (Fig. 5*B*). Both MAPK inhibitors prevented maximal induction by ~50%. Therefore, these cytoplasmic inhibitors have pleiotropic effects on nuclear hormone-mediated transcription.

The level of the ER itself may be decreasing in response to the inhibitory compounds, which could account for the decline in transcriptional activity. Therefore, we measured the levels of ER by immunoblotting over the course of a 24-h treatment regimen (Fig. 5*C*). ER is known to decline in response to E_2 treatment (37, 38); we observed this phenomenon with ER protein levels reduced 3-fold after 9 h of estrogen stimulation compared with basal levels in starved cells. Estrogen stimulation in the presence of LY294002, wortmannin, or PD098059 did not affect ER protein levels compared with E_2 alone. Furthermore, in the absence of estrogen, these drugs caused no change in the steady-state levels of ER found in serum starved cells. Consistent with published data, ICI 182,780 reduced ER levels in the presence or absence of steroid hormone (36). Unlike PD098059, treatment with U0126 decreased ER levels with kinetics similar to those observed with ICI 182,780. The diminishment of

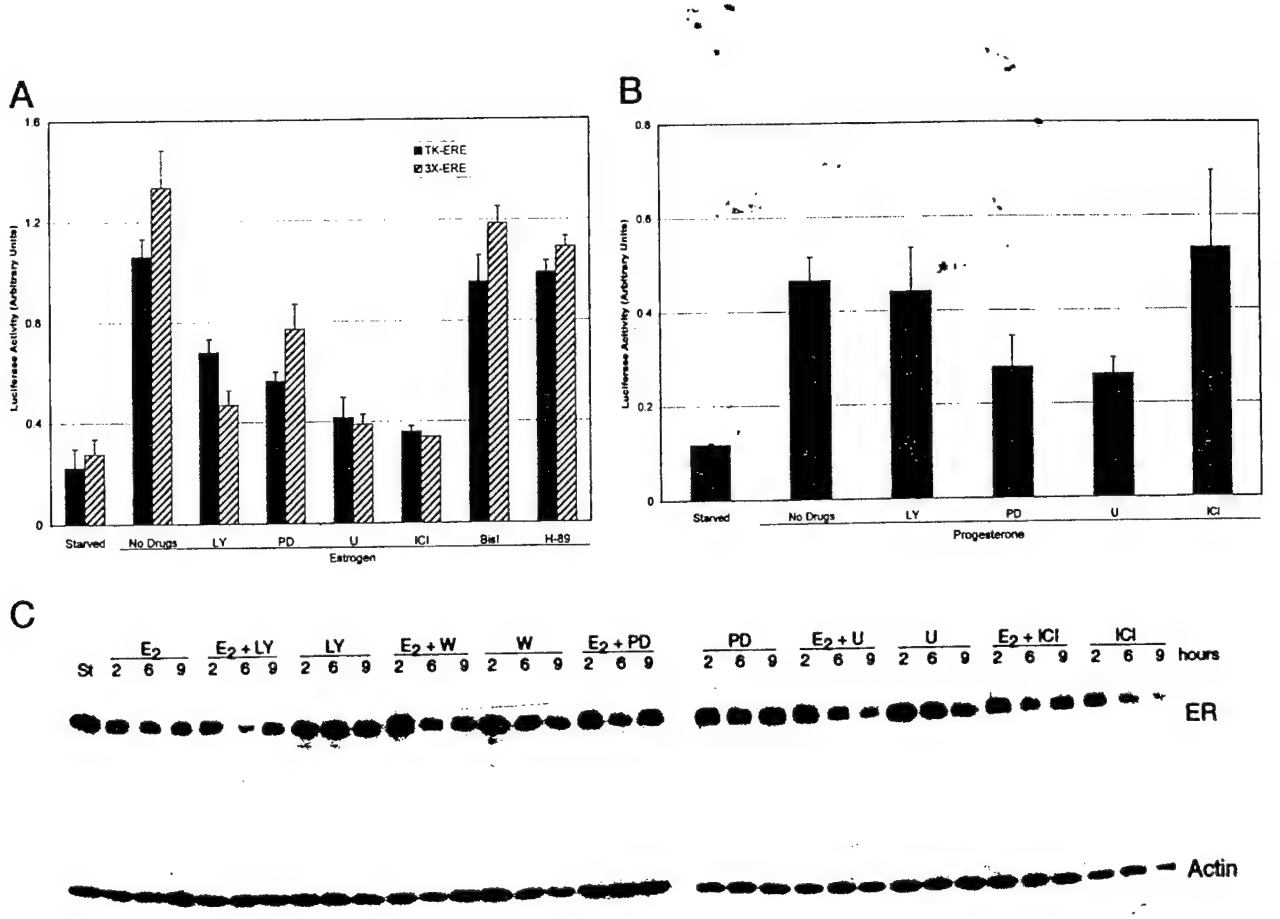

Fig. 5. Effect of cytoplasmic inhibitors on ER-dependent transcription. *A*, MCF-7 cells were transfected with a luciferase construct containing either an artificial (3X-ERE) or a naturally occurring (TK-ERE) estrogen-responsive promoter. Samples were harvested after 2, 6, 9, or 24 h of stimulation with 2×10^{-8} M E_2 in the presence or absence of 5 μM LY294002 (*LY*), 50 μM PD098059 (*PD*), 25 μM U0126)*U*), 2 μM ICI 182,780 (*ICI*), 750 nM bisindolylmaleimide I (*Bisl*), or 500 nM H-89, cells were lysed, and luciferase activity was assessed. The 2-h interval was not sufficient to generate detectable levels of luciferase activity; however, the 6- and 9-h time points displayed similar kinetics of induction and inhibition, as the 24-h results show. Corrections for differences in protein concentration and transfection efficiencies were made using detected levels of a cytomegalovirus-luciferase construct from a different species and are given for at least three experiments in triplicate; *bars*, SD. *B*, MCF-7 cells were transfected with a human progesterone receptor B expression construct in addition to a progesterone-responsive luciferase construct. Twenty-four h after treatment with 10^{-7} M progesterone and inhibitory compounds, cells were lysed, and luciferase activity was measured and corrected as detailed above. *Bars*, SD. *C*, cells were incubated with inhibitors in the presence or absence of 2×10^{-8} M E_2 for the times indicated prior to lysis. Western analysis was used to detect levels of ER (after 2, 6, and 9 h of stimulation). This blot was reprobed to quantitate actin levels to control for loading differences. *LY*, LY294002; *W*, wortmannin; *PD*, PD098059; *U*, 0126; *ICI*, ICI 182,780.

ER in the presence of U0126 and with ICI 182,780 is consistent with the dramatic inhibition of ER-mediated transcription at later time points.

Inhibitor Effects on Estrogen-induced Gene Transcription. The kinase inhibitors did exert a suppressive effect on estrogen-mediated transcription measured by reporter gene assay. These assays were performed between 6 and 24 h of drug treatment, long after the signaling cascades or the ER may transduce any immediate effects on the cell cycle. Decreased ER transcription may be a secondary effect of the arrest rather than a direct effect of kinase inhibitors. Expression of the immediate-early genes occurs rapidly after E_2 treatment; therefore, we measured the expression of these genes directly by Northern blotting. We observed maximal expression of the two putative estrogen-responsive immediate-early genes, c-*myc* and c-*fos*, within 1 h of E_2 treatment (Fig. 6). None of the signal transduction inhibitors (bisindolylmaleimide I, H-89, LY294002, and PD098059) had an effect on the kinetics or magnitude of estrogen-induced myc and fos expression (Fig. 6 and data not shown). Estrogen induction of myc and fos protein was also not inhibited by these compounds (data not shown). The ICI antiestrogen completely inhibited the induction of both c-*myc* and c-*fos* mRNA (and protein) as expected (Fig. 6 and data not shown). Despite decreasing the steady-state levels of ER, U0126 had no effect on the induction of myc and fos in response to estrogen treatment. Therefore, although the compounds did affect estrogen transcription measured in the transient assays, they had no effect on immediate-early gene synthesis. These results suggest that induction of these genes is directly dependent upon the hormone receptor's nuclear action. Furthermore, inhibition of estrogen transcription in the reporter gene assay at later times is not correlated with effects on the transcription of these endogenous genes.

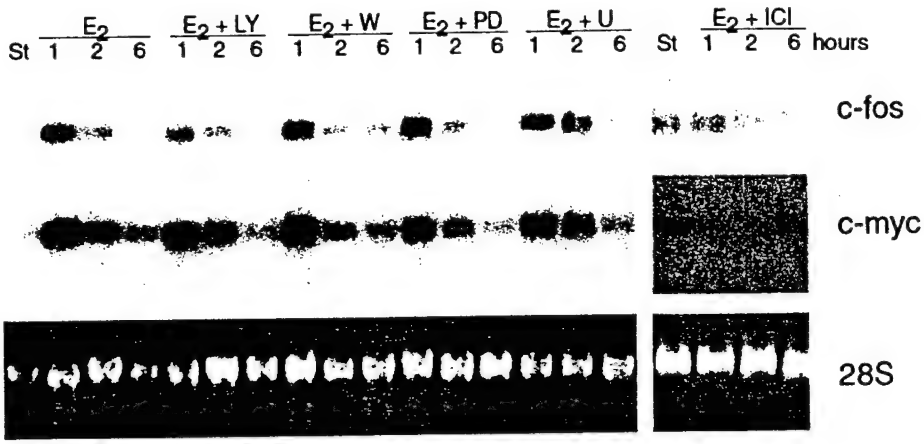

Fig. 6. Effect of cytoplasmic signaling inhibitors on E_2-induced immediate-early gene RNA levels. Representative Northern blot analysis of c-*myc* and c-*fos* RNA expression in MCF-7 cells treated with estrogen in the presence or absence of 5 μM LY294002 (*LY*), 100 mM wortmannin (*W*), 50 μM PD098059 (*PD*), 25 μM U0126 (*U*), or 2 μM ICI 182,780 (*ICI*; from a separate Northern blot) for the times (in hours) indicated for each lane. The starved lane displays the amount of c-*myc* or c-*fos* RNA present at time 0. Levels of the 28S ribosomal subunit were used as an internal control to correct for loading inconsistencies.

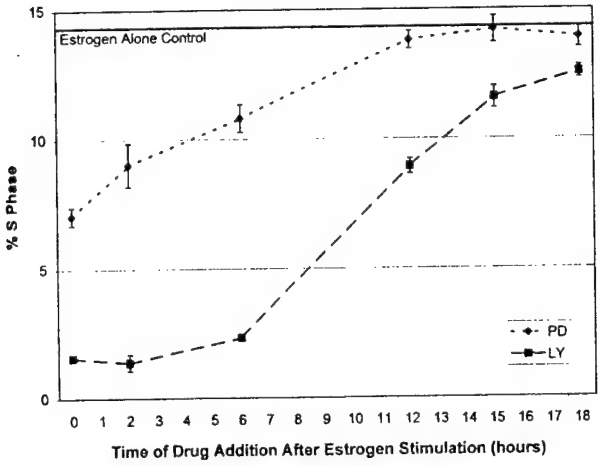

Fig. 7. Mitogenic effects of cytoplasmic signaling inhibitor addition after E_2 stimulation. Starved cultures of MCF-7 cells were treated with 2×10^{-8} M E_2 at time 0 and then treated with either 50 μM PD098059 (*PD*) or 5 μM LY294002 (*LY*) at 2, 6, 12, 15, or 18 h. At 24 h, cell cultures were harvested, and the fraction of cells in S phase was measured by flow cytometry. *Bars*, SD.

Timing of the Mitogenic Block. It is readily understandable how the ICI antiestrogen blocks cell cycle progression via inhibition of immediate-early gene expression (39, 40). However, the cytoplasmic signaling inhibitors did not prevent the immediate early genes from being induced, raising the question of at what stage of the cell cycle the block is imposed. We next investigated the timing of this inhibition relative to the mitogenic stimulus. Estrogen was added at time 0, and the inhibitors (PD098059 or LY294002) were added at varying times after the mitogenic stimulus. At 24 h, all cultures were harvested, and the percentage of cells in S phase was determined. We have shown previously that starved MCF-7 cells begin to enter S phase ~12 h after the addition of estrogen, with peak S phase values reached at 24 h (25). Effective inhibition of cell cycle progression was observed when LY294002 was added up to 6 h after exposure to estrogen (Fig. 7). By 12 h, the LY compound was no longer an effective inhibitor. In parallel experiments using the MEK1 inhibitor (PD098059), we observed a gradual increase in S-phase progression with delayed addition of the compound after E_2 stimulation. Although the demarcation in PD098059 activity was not as clearly defined, the profile from both compounds suggested that inhibition of events early in G_1 is important for the activity of these drugs in preventing S-phase progression.

Cyclin D1 Expression and Rb Phosphorylation in Response to Estrogen and Cytoplasmic Signaling Inhibitors. Increased cyclin D1 expression is considered to be a delayed-early event occurring after immediate-early genes are induced. Accumulation of cyclin D1 protein after E_2 stimulation is a critical event in G_1 progression (41). In addition, overexpression of cyclin D1 can reverse cell cycle arrest in ER-positive cells accomplished by antiestrogen treatment (42). These findings, coupled with our data showing that proliferation is inhibited by LY294002 only up to 6 h after E_2 stimulation, suggested to us that a delayed early event was being specifically inhibited. Therefore, we examined cyclin D1 expression and its downstream effects, CDK2 activity and subsequent Rb phosphorylation. Estrogen treatment of serum-starved MCF-7 cells resulted in a 2–3-fold increase in cyclin D1 protein levels by 6 h (Fig. 8*A*). This modest induction is consistent with other reports and with the 3–4-fold increase in S phase that we routinely observed under these conditions (41). As expected, cyclin D1 accumulation resulted in the activation of CDK2 and Rb hyperphosphorylation (Fig. 8, *B* and *C*; Ref. 43). The pharmacological compounds demonstrated a good correlation between antimitogenic activity and inhibition of the Rb pathway. The ICI antiestrogen, as reported previously (44), and the MAPK inhibitor U0126 both prevented cyclin D1 induction and activation of CDK2 and led to decreased levels of hyperphosphorylated retinoblastoma protein. These findings are consistent with the effects of ICI and U0126 on mitogenesis. Of the PI3-K inhibitors, wortmannin was not as potent at preventing the accumulation of cyclin D1 protein as LY294002, a finding that is consistent with the relative S-phase inhibition observed with these compounds. The MEK1 inhibitor PD098059 also inhibited cyclin D1 levels; however, the effect appeared to be more transitory as cyclin D1 began to accumulate at later time points (Fig. 8*A* and data not shown). This was also reflected by the incomplete inhibition of CDK2 activation, Rb hyperphosphorylation, and cell cycle progres-

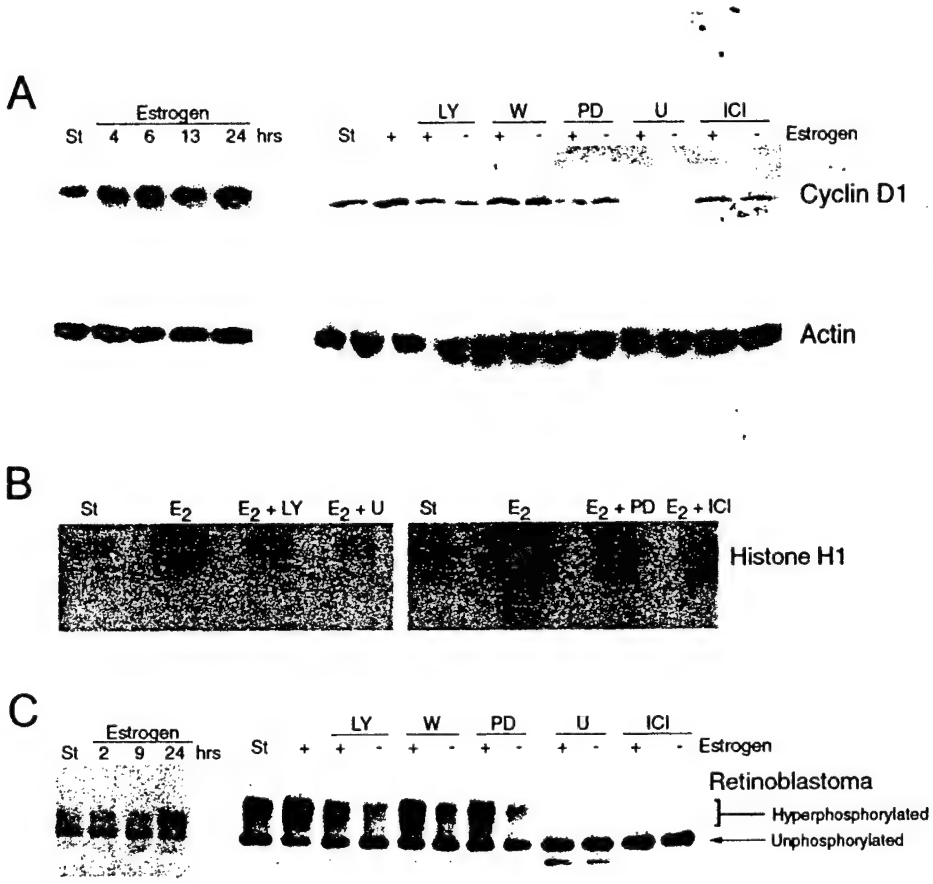

Fig. 8. Effects of cytoplasmic signaling inhibitors on cyclin D1 expression, CDK2 activity, and Rb phosphorylation. Cells were preincubated with 2×10^{-8} M E_2 in the presence or absence of 5 μM LY294002 (*LY*), 100 nM wortmannin (*W*), 50 μM PD098059 (*PD*), 25 μM U0126 (*U*), or 2 μM ICI 182,780 (*ICI*). *A*, after 6 h, the cells were lysed, and relative amounts of cyclin D1 and actin expression was detected by immunoblotting. *B*, after 24 h of treatment, cells were lysed, and CDK2 was immunoprecipitated; the activation status of this kinase was assayed by determining its ability to phosphorylate histone H1. *C*, Rb phosphorylation was assessed using Western analysis on cell lysates that were extracted after 24 h of stimulation.

sion. From these experiments, we place the critical antimitogenic effects of the PI3-K and MAPK inhibitors at or before the level of cyclin D1 induction.

Discussion

Estrogen, a nuclear hormone, exerts a mitogenic effect on MCF-7 cells without the addition of any other growth factors, which facilitates the study of this pathway in isolation. A great deal of work on estrogen-induced growth has led to several conclusions: (*a*) the response depends upon the presence of functional estrogen receptor; (*b*) immediate-early genes are transcriptionally activated; and (*c*) cyclin D1 is an important intermediate in inducing this cell cycle cascade (reviewed in Ref. 45). If one assumes that the primary mode of estrogen action is via its receptor acting as a ligand-inducible transcription factor, mitogenesis could be explained by the direct transcriptional activation of immediate-early genes that contain functional EREs. The recent reports of estrogen activation of cytoplasmic signaling components, particularly the MAPK pathway, complicate this relatively simple paradigm (11, 13, 15, 22–24, 46, 47). It is in this context that the current studies were performed.

We found that disruption of the PI3-K and MAPK cascades by specific inhibitors (PD098059, which inhibits MEK1; U0126, which inhibits MEK1 and MEK2; and LY294002 and wortmannin, which inhibit PI3-K) prevented or reduced the ability of MCF-7 cells to enter S phase in response to estrogen. These drugs arrested cells in G_1 and did not induce any significant cytotoxicity. We also demonstrated that E_2 does not measurably activate either PKB or MAPK under conditions that result in a mitogenic response. We did observe variable effects on estrogen-mediated transcription assayed on artificial EREs; however, the immediate-early genes c-*myc* and c-*fos* were both induced in the presence of these drugs. Both the PI3-K and MAPK inhibitors prevented cyclin D1 accumulation, which correlated with an absence of CDK2 activity and Rb phosphorylation. We concluded that inhibition of estrogen-mediated mitogenesis by these drugs was likely attributable to this repression of cyclin D1 expression, CDK2 activation, and Rb phosphorylation.

This study raises several issues regarding the mechanism of estrogen action and mitogenesis in general. The lack of any reproducible induction of MAPK activity, as measured by Erk1 and Erk2 phosphorylation, contradicts several published reports (13, 15, 21, 32, 47). In light of these reports, we examined this parameter many times using different time points, cell densities, and variable concentrations of charcoal-stripped serum. On occasion, we did observe a small increase (<1.5 fold) in Erk1 and Erk2 phosphorylation between 2 and 5 min after estrogen stimulation. Published accounts of this activation have also shown a relatively modest induction (between 1.5- and 3-fold) over control values (13, 47). In all reported cases and in our experiments, the magnitude of activated MAPK resulting from a cell surface signal such as EGF far exceeds any induction attributable to estrogen (32). Furthermore, estrogen can overcome a cell cycle block induced by Simvastatin, an HMG-CoA reductase inhibitor, without detectable MAPK activation (48). It is also

clear from our experiments that estrogen is mitogenic in the absence of measurable MAPK activation, which raises the issue of the physiological relevance of the response when it is observed.

MAPK and PI3-K inhibitors were shown to inhibit ER-mediated transcription, assayed using a gene reporter system. However, these inhibitors do not prevent the expression of immediate-early genes in response to estrogen. At least one plausible explanation can reconcile this apparent discrepancy. Maximal expression of c-*myc* and c-*fos* occurs after 1 h of stimulation, whereas luciferase activity required 6 h for a measurable response. Therefore, effects on ER transcription may not be manifest until after the rapid immediate-early response. How critical this delayed inhibition of ER activity is in mediating the growth arrest remains to be determined.

Estrogen stimulates the production and secretion of growth factors that can function in an autocrine fashion (49). For example, protein levels of fibroblast growth factor-1 increase in response to E_2 treatment in MCF-7 cells and may act synergistically with the hormone to generate a greater proliferative response (50, 51). Fibroblast growth factor-2 is also up-regulated in response to E_2 stimulation of human endothelial cells and is required for the MAPK activation that is observed 3 h after exposure to estrogen (52). Continuous incubation in the presence of MEK inhibitors would prevent the activation of MAPK by growth factors acting in an autocrine loop. Therefore, the cell cycle block observed with PD098059 and U0126 may be a result of inhibiting autocrine cascades necessary to realize the mitogenic potential of estrogen. Our finding that an antiproliferative response can still be achieved when adding the inhibitory drugs up to 6 h after hormone stimulation further substantiates this theory.

Our results strongly suggest that cyclin D1 induction is a key intermediate in estrogen mitogenesis. Several reports have focused on the role of cyclin D1 activation in estrogen-mediated mitogenesis. MCF-7 cells overexpressing cyclin D1 are unable to proliferate in the presence of antiestrogens (53). Planas-Silva and Weinberg (41) demonstrated that estrogen relieves a cell cycle block in tamoxifen arrested MCF-7 cells by increasing levels of cyclin D1 protein, which causes the redistribution of $p21^{cip1/waf1}$ away from the cyclin E/CDK2 complexes, allowing Rb phosphorylation and cell cycle progression. Prall *et al.* (42) derived MCF-7 cell lines with inducible expression of c-*myc* or cyclin D1. Cell cycle arrest of these lines by the estrogen antagonist ICI 182,780 was reversed by expression of either myc or cyclin D1. In both cases, loss of the CDK inhibitor p21 from cyclin E/CDK2 complexes was noted. Interestingly, c-*myc* overexpression did not result in elevated cyclin D1 levels, and when cyclin D1 was induced, myc levels remained constant, implicating the existence of multiple pathways to remove p21. Our data indicate that c-*myc* induction is not sufficient to allow estrogen-mediated cell cycle progression in the face of diminished cyclin D1 expression.

Functional activity of ectopically expressed ER is not sufficient for the expression of cyclin D1 and subsequent proliferation in response to estrogen (54). However, Castoria *et al.* (21) reported recently that fibroblasts transiently transfected with a transcriptionally inactive ER (lacking the DNA binding domain) exhibited E_2-dependent cell cycle progression, presumably attributable to the ability of the receptor to activate the MAPK cascade. This report suggests that proliferation in response to estrogen may be a result of nonnuclear events rather than ER-mediated transcription. In contrast, in a host of other studies, ectopic expression of active ER (measured by transcriptional activity) is not sufficient to convert cells into E_2 responders (55–61). Our data indicate that E_2-induced proliferation can occur in the absence of detectable MAPK activation. Although other studies have shown E_2-induced MAPK activity, the link between MAPK and mitogenesis after E_2 stimulation has not been established. Specific culture conditions, experimental conditions, and divergence of cells in long-term culture may explain the discrepant results.

The question raised by our data is how the inhibitors of cytoplasmic signaling prevent S phase although the targets of these inhibitors are apparently not activated. Repression of cyclin D1 may be the key to understanding these observations. The molecular mechanism by which the PI3-K cascade functions in cyclin D1 accumulation has begun to be characterized as a posttranscriptional phenomenon. In the absence of mitogen, cyclin D1 is targeted for ubiquitin-dependent degradation because of the presence of a phosphate group at threonine 286, a substrate for GSK-3β (62, 63). Inactivation of GSK-3β occurs as a result of PKB activation, a downstream target of the PI3-K cascade (Ref. 44 and reviewed in Ref. 64). Thus, the cell cycle block induced by LY294002 in the presence of E_2 may be attributable to the inability of GSK-3β to be inactivated by PKB, thereby preventing the accumulation of cyclin D1.

The role of MAPK in cyclin D1 accumulation is less well defined. Typically, activation of MAPK results in nuclear translocation and activation of immediate-early gene expression. Several studies, however, do suggest a more direct effect. Using a Chinese hamster fibroblast cell line, CCL39, Lavoie *et al.* (65) demonstrated that not only was MAPK activation required for expression of endogenous cyclin D1, but also sufficient for full induction of this protein in the absence of growth factors. One theory to explain this phenomenon is that activated MAPK phosphorylates PHAS-1, which in turn impacts mRNA cap recognition and translation via eIF-4E (66). Therefore, inhibition of these cascades may prevent cyclin D1 accumulation at the level of mRNA utilization.

We propose that whereas estrogen may directly impact some components of cytoplasmic signaling cascades, the primary mode of action is within the nucleus. The finding that MAPK and PI3-K inhibitors prevent estrogen-induced mitogenesis indicates that cytoplasmic events are still required in this process. This underscores the importance of determining the molecular events required for the accumulation of cyclin D1 protein and the role of estrogen in autocrine loops, which may stimulate signaling cascades.

Materials and Methods

Materials. ICI 182,780 was purchased from Tocris. E_2, EGF, IGF-I, LY294002, wortmannin, bisindolylmaleimide I, and H-89 (dihydrochloride)

were obtained from Calbiochem. PD098059 was obtained from New England Biolabs. A monoclonal antibody against diphosphorylated Erk1 and Erk2 was purchased from Sigma Chemical Co.; polyclonal antibodies against Erk2 (clone K-23), ERα (G-20), CDK2 (M-2), the monoclonal antibody recognizing cyclin D1 (HD11), and normal goat IgG were obtained from Santa Cruz Biotechnology. Actin (C4) monoclonal antibody and protein G-agarose beads were from Boehringer Mannheim, and the monoclonal antibody against Rb protein (G3–245) was purchased from PharMingen. Polyclonal antibodies against PKB and Ser-473-phosphorylated PKB were obtained from New England Biolabs. Donald P. McDonnell kindly provided the estrogen-responsive luciferase constructs (3X-ERE and TK-ERE), the progesterone-responsive luciferase construct (3X-PRE), and the human progesterone receptor expression plasmid.

Cell Culture. MCF-7 cells were obtained from American Type Culture Collection and propagated in RPMI 1640 containing 10% (v/v) FBS (Life Technologies, Inc.). ER content was measured using the ERICA kit (Abbot Labs, Inc.) and quantitated using the CAS 200 Image Analyzer (Cell Analysis Systems, Inc.). MCF-7 cells express 170 fmol/mg of protein as compared with <10 fmol/mg of protein found in normal breast epithelial cells.

For experimental purposes, cells were seeded in six-well format at a density of 3×10^5 cells/well or in a 60-mm dish at a density of 5×10^5 cells. After 24 h, cells were washed one time with phenol red-free RPMI without FBS. Cells were then incubated for an additional 24 h in this medium to effect growth factor deprivation (67).

Cell Cycle Analysis/Viability. After 24 h of stimulation with 2×10^{-8} M E_2 in the presence or absence of inhibitory drugs, cells were trypsinized and pelleted prior to washing once in PBS. Cell pellets were fixed in 70% ethanol (in PBS) overnight on ice. Cells were washed with PBS and resuspended in 500 μl of PBS supplemented with 100 μg of RNase A and 50 μg of propidium iodide. DNA content was determined on a per cell basis using flow cytometry. Cell viability was assessed on trypsinized cells by staining with erythrosin B and determining the percentage of viable cells (500 cells counted) by microscopic examination in three separate experiments.

Northern Blotting. Total RNA was extracted from 5×10^5 cells using the Trizol reagent according to the manufacturer's instructions (Life Technologies). Five μg of RNA were electrophoresed on a 1% agarose-2.2 M formaldehyde gel and then transferred onto a nylon membrane (ICN). Blots were cross-linked using UV irradiation and hybridized with a ^{32}P-labeled probe prepared by random priming (cDNA plasmid clones were purchased from American Type Culture Collection). Blots were washed for 10 min at 65°C with 2× SSC (150 mM NaCl and 15 mM $Na_3C_6H_3O_7$, pH 7.0) containing 1% SDS and then with 0.1× SSC/0.1% SDS prior to autoradiography at −80°C with intensifying screens. IMAGEQuant (Molecular Dynamics) was used to quantify the expression levels of the gene of interest, and loading inconsistencies were corrected using detected levels of the housekeeping gene, *glyceraldehyde-3-phosphate dehydrogenase*.

Transient Transfections/Luciferase Assay. For liposome delivery, cells were washed with serum-free medium (Opti-Mem I; Life Technologies). The firefly luciferase gene (*Photinus pyralis*), under the control of estrogen- or progesterone-responsive promoters (artificial or from the human vitellogenin gene) as well as a cytomegalovirus-driven luciferase construct (*Renilla reniformis*) were combined (2.5 μg/ml and 5 ng/ml, respectively) prior to transfection, following the standard protocol for Lipofectin (Life Technologies). (Because of low endogenous expression levels of progesterone receptor in MCF-7 cells, a human progesterone receptor B expression construct was also included in the transfection mix for experiments using progesterone). Cells were incubated with the liposome solution for 5 h, at which time the medium was aspirated and replaced with RPMI 1640 without phenol red, supplemented with 10% (v/v) charcoal-stripped FBS. Twenty-four h later, cells were treated with 2×10^{-8} M estrogen in fresh medium, either in the presence or absence of inhibitory drugs. Cells were lysed after 24 h of stimulation, and luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega) and a 20/20 dual-channel luminometer (Turner Designs).

Immunoblotting. Cells were lysed in 2× SDS sample buffer [25 mM Tris-HCl (pH 6.8), 12.5% glycerol, 6% SDS, 0.045% bromphenol blue, 4% β-mercaptoethanol, and 1 mM sodium orthovanadate]. Protein separation was achieved using 10% SDS-PAGE and electrotransferred to nitrocellulose membranes (Schleicher & Schuell). To prevent nonspecific interactions of the antibody, the blots were blocked for 1 h at room temperature with PBS containing 0.01% Tween 20 (PBS-T) and 5% nonfat dry milk. Membranes were incubated with primary antibodies in PBS-T milk, at recommended dilutions, overnight at 4°C. Antigen-antibody complexes were visualized by incubation with horseradish peroxidase-conjugated goat antirabbit or antimouse IgG (Jackson Laboratories) for 1 h at room temperature, followed by ECL detection (DuPont NEN Life Science). Quantitation of protein levels was performed as detailed above, with actin levels being used to correct for variable loading.

CDK2 Kinase Assay. After 24 h of estrogen stimulation in the presence or absence of inhibitory compounds, cells were lysed in NP40 lysis buffer [50 mM Tris (pH 8), 5 mM EDTA, 150 mM NaCl, and 0.5% NP40] on ice for 30 min. Insoluble proteins were removed by centrifugation, and 500 μg of the remaining lysate were precleared with 1 μg of normal goat IgG and 20 μl of protein G-agarose beads. Precleared lysate was immunoprecipitated with an anti-CDK2 antibody and immunocomplexes were washed twice with lysis buffer and once with kinase buffer [50 mM Tris (pH 7.5), 10 mM $MgCl_2$, and 1 mM DTT]. Five μg of histone H1, 1 μM dATP, and 5 μCi [γ^{32}P]dATP in 50 μl of kinase buffer were added to the beads prior to a 30-min incubation at 30°C. The reaction was terminated with the addition of 10× SDS sample buffer, and the samples were separated using 15% SDS-PAGE. The gel was dried and exposed to X-ray film.

Acknowledgments

We thank Donald P. McDonnell for the generous gift of the 3X-ERE, TK-ERE, 3X-PRE, and hPR-B constructs; Lenora Q. Blount for invaluable assistance in cell culturing; Carey J. Oliver for helpful discussions and comments; and Rita Nahta for critical review of the manuscript.

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